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Developmental and sex differences in kappa opioid receptor regulation of nicotine and alcohol responses

DISSERTATION

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for the degree of

DOCTOR OF PHILOSOPHY

in Biomedical Sciences

by

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DEDICATION

To my Nana, Simone Marie
Pour votre amour et votre soutien

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ABSTRACT OF THE DISSERTATION

Developmental and sex differences in kappa opioid receptor regulation of nicotine and alcohol responses

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Alcohol and nicotine, either from tobacco or increasingly from electronic cigarettes, are the two most commonly co-used drugs of abuse. Dual use has increased over time among teenagers and young adults, and represents a major public health concern among adolescents. During adolescence, the brain undergoes a profound reorganization in areas critical for executive function, reward processing, and motivated behavior that are necessary for adult autonomy, but do leave the adolescent brain vulnerable to the deleterious effects of drugs of abuse, including nicotine and alcohol. Neurobiological mechanisms mediating high rates of co-use are not well understood. However, as shown in this dissertation, developmental and sex-dependent changes in the dynorphin/kappa opioid receptor (KOR) system likely contribute.

In this dissertation, I emphasize the profound complexity of the KOR system in modulating acute drug- and mood-associated behaviors across development and between sexes. The present studies use behavioral pharmacological approaches to demonstrate that KOR activity differentially modulates the reinforcing properties of

nicotine and/or alcohol. Anatomical studies suggest that distinct, drug-specific anatomical pathways containing KORs modulate the reinforcing properties of these drugs. I have also shown that drug-induced versus direct KOR activation differentially influences mood-associated behaviors. Concurrent nicotine and alcohol (Nic+EtOH) produced a KOR-dependent decrease in anxiety-like behavior in adult females that was paralleled by increased KOR function in the basolateral amygdala. In contrast, two selective KOR agonists exerted a robust anxiogenic effect in this group, that may suggest distinct circuitry mediating direct versus indirect KOR activation on anxiety. Both adolescent and adult males displayed increased anxiety following KOR agonist treatment, while only adolescent males were sensitive to the pro-depressant effects of direct KOR activation, and Nic+EtOH had a subtle anxiolytic effect in this group. I also demonstrate a unique and previously unknown immaturity of KORs in adolescent females, as they were largely impervious to the effects of KOR manipulation on drug- or mood-associated behaviors. As a whole, my work emphasizes the profound complexity of KOR signaling and highlights important maturational and sex-dependent changes in KOR function. These changes may act as a critical, but underrecognized, influence of nicotine and alcohol co-use among teenagers and women.

Chapter 1

Introduction

I. Background and significance

Alcohol and nicotine, from tobacco and increasingly from electronic cigarettes, are the two most commonly co-used drugs of abuse. Smoking is common in individuals with alcohol use disorder (AUD; Batel *et al.*, 1995; Falk *et al.*, 2006; Moss *et al.*, 2015; Weinberger *et al.*, 2019), and dependent tobacco users are about ten times more likely to have comorbid AUD diagnoses than non-smokers (DiFranza & Guerrera, 1990). Even nondependent drinkers are more likely to smoke than non-drinkers (Kandel *et al.*, 1997). Dual use of nicotine and alcohol represents a major public health concern given the magnitude of affected individuals, substantial economic burden, and severe health consequences. In the United States alone, approximately six million people have comorbid nicotine and alcohol use disorders (Falk *et al.*, 2006), and economic costs are estimated to total nearly 500 billion dollars annually (NIDA, 2015). Concurrent use is associated with multiple negative health outcomes, including less success with cessation (Humfleet *et al.*, 1999; Kahler *et al.*, 2009; Weinberger *et al.*, 2017) and an increased risk of developing cancer, heart disease, mood disorders, and further substance abuse or dependence (Blot *et al.*, 1988; Le Strat *et al.*, 2010; Rueda *et al.*, 2012; Dal Maso *et al.*, 2015; MacLean *et al.*, 2018). However, mechanisms mediating high rates of co-use and co-abuse are not well understood.

Nicotine as a "gateway" drug

One potential mechanism for these associations is developmental exposure to nicotine. Epidemiological data suggest that exposure to nicotine, particularly during adolescence, can act as a "gateway" to further substance use, including alcohol (Kandel *et al.*, 1992; Lai *et al.*, 2000; Degenhardt *et al.*, 2010; Kandel & Kandel, 2015). Indeed, almost 90% of current adult smokers began smoking before the age of 18 (SAMHSA, 2011), and alcohol is typically first consumed before the age of 16 (Behrendt *et al.*, 2009). Even when young adults only smoke occasionally, they are very likely to drink, and consume significantly more alcohol when drinking and smoking occur together (Weitzman & Chen, 2005; Campbell *et al.*, 2012). Teenage drinking is associated with higher rates of AUDs (Grant & Dawson, 1997; DeWit *et al.*, 2000; Behrendt *et al.*, 2009) and smokers who start as teens consume more tobacco, are more likely to develop dependence, and have a harder time quitting than those who started as adults (Breslau & Peterson, 1996; Grant, 1998; Kandel & Chen, 2000; Riala *et al.*, 2004; Weitzman & Chen, 2005). Although a recent assessment of 12th grader patterns of use from 1976-2010 has shown that overall drug use has declined among this population, tobacco and alcohol co-use has increased over time (Daw *et al.*, 2013). Taken together, these epidemiological data support an important relationship between adolescent tobacco or nicotine use and severe, lasting consequences on substance use and dependence.

E-cigarettes as an emerging public health problem

Although the use of traditional cigarettes is declining (Farrelly *et al.*, 2013), the use of electronic cigarettes, or e-cigarettes, is rising rapidly (Camenga *et al.*, 2014;

Cullen *et al.*, 2018). Originally marketed as cessation agents and a healthier alternative to traditional tobacco cigarettes, data are inconsistent regarding the efficacy of e-cigarettes as cessation aids (Hartmann-Boyce *et al.*, 2016; Malas *et al.*, 2016; Hajek *et al.*, 2019; Piper *et al.*, 2019), and their use by teenagers and young adults has increased exponentially in recent years (Camenga *et al.*, 2014; Cullen *et al.*, 2018). In fact, e-cigarette use has recently surpassed traditional cigarette use among teens (Miech *et al.*, 2015a; Jamal *et al.*, 2017). As a result, e-cigarettes represent an emerging public health problem.

The health consequences of e-cigarette use are not well understood, particularly among adolescents. Notably, adolescents are less likely to attribute significant potential harm to e-cigarettes (Gorukanti *et al.*, 2017; McKelvey *et al.*, 2018), and their use may act as a "gateway" to traditional tobacco cigarette use (Lanza *et al.*, 2017; McCabe *et al.*, 2017). Among young adult tobacco smokers, concurrent e-cigarette use is associated with higher smoking rates and odds of tobacco use disorder, as well as less success with cessation (Olfson *et al.*, 2019). E-cigarette use has also been linked to increased alcohol consumption, binge drinking, and AUD among teenagers and young adults (McCabe *et al.*, 2017; Roberts *et al.*, 2018; Hefner *et al.*, 2019; Thrul *et al.*, 2019). These effects may be exacerbated by concurrent e-cigarette and tobacco cigarette use (Roberts *et al.*, 2018; Olfson *et al.*, 2019). Despite these strong associations, the neurobiological consequences of developmental exposure to nicotine and alcohol are not well understood.

Overall, concurrent nicotine and alcohol use poses a significant public health problem, particularly for teenagers and young adults whose use continues to rise. Neither the neurobiological mechanisms nor consequences are well understood. The following literature review describes the dynamic maturation of the adolescent brain, and highlights the dynorphin/kappa opioid receptor system as a potential neurobiological substrate contributing to high rates of nicotine and alcohol co-use during this developmental period.

II. What is adolescence?

Adolescence is an evolutionarily conserved transition period between childhood and adulthood that is conservatively estimated to last from 12 to 18 years of age in humans and postnatal (P) days 28-42 in rodents (Spear, 2000). Adolescents display characteristic behaviors of increased risk-taking, novelty seeking, and peer associations that are thought to ease the transition to adult autonomy (Spear, 2000; Spear, 2013). However, the adolescent brain is uniquely vulnerable to the effects of drugs of abuse, including nicotine and alcohol (Chambers *et al.*, 2003; Crews *et al.*, 2007).

Adolescence is marked by major reorganization of brain regions involved in cognitive and executive function, learning and memory, emotional regulation, and reward processing (Spear, 2000; Yuan *et al.*, 2015). Subcortical emotional and reward-focused regions mature earlier than cortical executive and impulse control systems, producing an imbalance thought to underlie characteristic adolescent behaviors (Cunningham *et al.*, 2002; Brenhouse *et al.*, 2008; Ernst & Fudge, 2009; Casey *et al.*, 2011; Smith, 2013). Connections from the prefrontal cortex (PFC) to the nucleus accumbens (NAc) and from the amygdala to the PFC actively mature (Cunningham *et*

al., 2002; Brenhouse *et al.*, 2008), and these connections play an important role in cognition, mood, reward, and motivated behavior (Giedd, 2004; Gogtay *et al.*, 2004; Heyder *et al.*, 2004; Ernst & Fudge, 2009). Synaptic pruning in the PFC, amygdala, and striatum also increases (Teicher *et al.*, 1995; Andersen *et al.*, 2000; Zehr *et al.*, 2006), with PFC pruning continuing into early adulthood, resulting in late maturation of higher-order cognitive functions (Giedd, 2004; Cressman *et al.*, 2010).

In addition to the structural changes that occur in the adolescent brain, there are substantial neurochemical changes. The dopamine system, which plays a critical role in reward processing and motivated behaviors, undergoes a profound reorganization that extends into early adulthood (O'Donnell, 2010; Wahlstrom *et al.*, 2010). Dopamine receptor binding sites exhibit a pattern of overproduction followed by region- and cell-type specific pruning that is more robust in males (Teicher *et al.*, 1995; Andersen *et al.*, 1997; Brenhouse *et al.*, 2008; Naneix *et al.*, 2012). In the NAc, D1 and D2 receptor responses are immature, leading to decreased synaptic interaction between this region and the PFC (Benoit-Marand & O'Donnell, 2008). Dopamine innervation undergoes a late maturation, particularly in the anterior PFC (Cao *et al.*, 2007b; Naneix *et al.*, 2012). Firing of ventral tegmental area (VTA) dopamine neurons is higher in adolescents than adults (Placzek *et al.*, 2009; McCutcheon *et al.*, 2012), and neurotransmitter turnover in target regions is greater (Tarazi *et al.*, 1998; Moll *et al.*, 2000; Naneix *et al.*, 2012). Extracellular levels of dopamine peak in late adolescence in the NAc (Philpot *et al.*, 2009), and psychostimulant-induced dopamine release in the striatum undergoes late maturation (Cao *et al.*, 2007b; Matthews *et al.*, 2013). Importantly, phasic dopamine firing in adolescents, but not adults, aids in the formation of mesofrontal axonal boutons,

resulting in greater mesofrontal circuit activity (Mastwal *et al.*, 2014). Taken together, these studies highlight the late-maturing dopamine system as a locus for the particular vulnerability of adolescents to drug-induced alterations.

The serotonin system, which is also involved in reward processing and motivated behaviors, undergoes a less extensive reorganization during adolescence than dopamine, but seems to be particularly sensitive to drug-induced perturbation (Shearman *et al.*, 2008; Bang & Commons, 2011; Dao *et al.*, 2011; Slotkin *et al.*, 2014). Adolescence is associated with increased innervation and pruning of serotonin projections (Morilak & Ciaranello, 1993), as well as a gradual increase in levels of serotonin and the serotonin transporter in limbic regions (Tarazi *et al.*, 1998; Dao *et al.*, 2011). As a result, drugs that affect serotonin signaling may have more profound, long-lasting effects on the adolescent brain (Xu *et al.*, 2002; Shearman *et al.*, 2008; Slotkin & Seidler, 2009; Dao *et al.*, 2011; Slotkin *et al.*, 2014).

Other neurotransmitter systems, particularly those that exert a modulatory influence over dopaminergic and serotonergic signaling, may also actively mature during adolescence. Indeed, as reviewed in detail later, the dynorphin and kappa opioid receptor (KOR) system negatively regulates dopamine and 5-HT activity and appears to undergo an important functional maturation during adolescence.

III. Nicotine and alcohol share genetic and neurobiological mechanisms

High rates of nicotine and alcohol co-use may be explained, in part, by overlapping neurobiological and genetic mechanisms. A large body of evidence in both humans and rodents has demonstrated that consumption of one drug can influence consumption of the other. In humans, alcohol-associated cues and fixed-dose alcohol

administration can elicit cravings to smoke in both nondependent and AUD individuals, although craving severity is greater in the latter group (Rohsenow *et al.*, 1997; Drobes, 2002; McGrath *et al.*, 2015). Reciprocal effects, with nicotine cues or smoking increasing alcohol craving, have also been reported (Gulliver *et al.*, 1995; Drobes, 2002).

As in humans, there are positive relationships between nicotine exposure and alcohol intake or self-administration in rodents (Lê *et al.*, 2000; Olausson *et al.*, 2001; Lê *et al.*, 2003; Bito-Onon *et al.*, 2011; Hauser *et al.*, 2012; Doyon *et al.*, 2013a), although this can depend on length and timing of exposure, strain, route of administration, and sex. Single systemic injections of nicotine have no effect on alcohol intake, while repeated exposure enhances both oral ethanol intake and self-administration in males (Lê *et al.*, 2000; Olausson *et al.*, 2001; Lê *et al.*, 2003; Bito-Onon *et al.*, 2011). A recent study, however, found that systemic nicotine injections dose- and sex-dependently alter ethanol reinforcement, with low doses (0.05-0.2 mg/kg nicotine) enhancing oral ethanol self-administration in females, but not males (Barrett *et al.*, 2019). This may suggest that females are more sensitive to the reinforcement enhancing effects of nicotine, although direct comparisons between sex are limited.

Neurobiological substrates underlying unique behavioral effects of concurrent versus individual nicotine and alcohol exposure are limited, but implicate midbrain and limbic regions. Increased alcohol drinking 3-4 hours after nicotine exposure (Hauser *et al.*, 2012; Doyon *et al.*, 2013a) is accompanied by greater GABAergic inhibition of VTA neurons, resulting in decreased dopamine cell firing and lower NAc dopamine release that could have increased the motivation for ethanol (Doyon *et al.*, 2013a; Doyon *et al.*,

2013b). An alternative hypothesis suggests that greater reward is due to nicotine and alcohol having additive or synergistic effects within the mesolimbic dopamine system. Indeed, systemic nicotine plus intra-accumbens ethanol increases dopamine levels more than either drug alone (Tizabi *et al.*, 2002; Tizabi *et al.*, 2007) and nicotine pretreatment into the VTA enhances ethanol's stimulatory effects on dopamine release (Ding *et al.*, 2012). Furthermore, both male and female alcohol preferring (P) rats self-administer combined nicotine and alcohol into the posterior VTA (pVTA) at concentrations that do not support individual drug self-administration (Truitt *et al.*, 2015), highlighting the pVTA as a key locus for enhanced reinforcement of combined nicotine and alcohol. Together, these data illustrate that prior nicotine can influence ethanol intake, and highlight unique effects of concurrent versus individual nicotine and alcohol exposure that may be mediated through mesolimbic structures such as the pVTA.

Preclinical evidence for nicotine's "gateway" effects

Increasing preclinical data lend support to the "gateway" theory of drug addiction (McQuown *et al.*, 2007; McQuown *et al.*, 2009; Dao *et al.*, 2011; Kandel & Kandel, 2015; Alajaji *et al.*, 2016; Lárraga *et al.*, 2017; Kota *et al.*, 2018). Despite strong epidemiological associations between age of nicotine exposure and subsequent substance use, age is not consistently taken into account in preclinical research on nicotine and alcohol. A growing body of literature, however, shows that nicotine and alcohol produce unique behavioral and neurochemical responses in adolescents. Our lab has demonstrated that concurrent acetaldehyde, the primary metabolite of ethanol, increases initial acquisition of nicotine self-administration in adolescent male rats

(Belluzzi *et al.*, 2005). As adolescence proceeds, males exhibit a decrease in responding to nicotine-acetaldehyde combinations, while females do not (Park *et al.*, 2007). Periadolescent nicotine (P35-44) enhances ethanol consumption in female mice (Locker *et al.*, 2015) and adolescent nicotine self-administration increases oral ethanol intake in late adolescence (Lárraga *et al.*, 2017), although one study reported no effect of adolescent nicotine exposure on ethanol intake in adulthood (Smith *et al.*, 2002).

We have also shown that brief, low-dose nicotine pretreatment during early adolescence (P28-31) enhances acquisition of alcohol self-administration (Dao *et al.*, 2011). Recent work further demonstrates that self-administration of nicotine during adolescence, alone or in combination with ethanol, increases subsequent oral alcohol intake (Lárraga *et al.*, 2017). Further, concurrent intravenous nicotine and ethanol (Nic+EtOH) is more reinforcing in adolescent males compared to adults (Lárraga *et al.*, 2017). This enhanced reinforcement of co-administered drugs is not evident in adult males or females, and seems to result from a functionally immature kappa opioid receptor (KOR) system.

IV. The dynorphin and kappa opioid receptor system

The KOR and its endogenous ligand dynorphin A (Chavkin *et al.*, 1982) are widely distributed in the brain, particularly in regions involved in motivation and reward, mood, and stress responsiveness (Kornblum *et al.*, 1987; Mansour *et al.*, 1995). The distribution of KORs overlaps substantially with dopaminergic and serotonergic systems, thus, poising them to be critical modulators of mood- and reward-related behaviors.

As with other opioid receptors, KOR activation produces analgesia. However, KORs are distinct from mu and delta opioid receptors in that activation by dynorphin or selective agonists also results in dysphoria, aversion, and anxiety- or depressive-like effects (Pfeiffer *et al.*, 1986; Van't Veer & Carlezon, 2013; Lalanne *et al.*, 2014). These effects have been attributed to intracellular signal bias (Bruchas *et al.*, 2007; Bruchas & Chavkin, 2010; Bruchas *et al.*, 2011; Chavkin *et al.*, 2014; Ehrich *et al.*, 2015; Abraham *et al.*, 2018) and inhibition of monoamine neurotransmitters. Indeed, systemic or intra-NAc KOR agonists lower dopamine levels in the NAc through actions on KORs located on presynaptic dopamine terminals (Spanagel *et al.*, 1992; Bals-Kubik *et al.*, 1993) and through direct interactions with the dopamine transporter (DAT; Svingos *et al.*, 2001). Following drug exposure, KOR's negative regulation of dopamine activity is thought to produce a compensatory decrease in reward state that inhibits subsequent intake (Spanagel *et al.*, 1992; Chefer *et al.*, 2005; Zapata & Shippenberg, 2006). Serotonin signaling is also inhibited by KOR activity through similar mechanisms (Tao & Auerbach, 2002; Bruchas *et al.*, 2011; Sundaramurthy *et al.*, 2017). Interestingly, selectively ablating KORs in dopamine neurons under the control of the DAT promoter, or serotonin neurons in the dorsal raphe, prevents KOR-mediated aversion (Land *et al.*, 2009; Bruchas *et al.*, 2011; Chefer *et al.*, 2013). These data highlight the interdependency of monoamine and KOR systems in regulating mood- and drug-associated behaviors.

<u>KORs and substance use</u>

Given the substantial overlap between mesocorticolimbic monoamine and dynorphin/KOR pathways, it is not surprising that KOR signaling has been implicated in drug dependence. In humans, genetic mechanisms link the KOR system and alcohol dependence, as SNPs in intron 2 of the KOR gene, *oprk1*, are significantly associated with alcoholism (Xuei *et al.*, 2006). Other variants in *oprk1*, including indels upstream of the transcription start site and the GGCTTCT haplotype, have also been positively associated with AUD (Edenberg *et al.*, 2008; Zhang *et al.*, 2008; Li & Zhang, 2013; Wang *et al.*, 2014), although some discrepancies are present (Cupic *et al.*, 2013; Karpyak *et al.*, 2013). Smoking and tobacco dependence, however, are associated with variants in the mu opioid receptor gene, *oprm1* (Norman & D'Souza, 2017).

Extensive research in rodents emphasizes the dynorphin/KOR system's important modulatory influence on motivated behavior. However, these findings are incredibly complex and vary widely based on drug, timing, and behavioral measure. Age and sex are also critical, yet understudied, factors regulating KOR function. Genetic studies show that global knockout of KORs is associated with lower oral alcohol self-administration (Kovacs *et al.*, 2005). Pharmacological studies report that KOR agonists can reduce the reinforcing and rewarding effects of cocaine, heroin, morphine, and alcohol (Kuzmin *et al.*, 1997; Nestby *et al.*, 1999; Lindholm *et al.*, 2001; Logrip *et al.*, 2009; Morani *et al.*, 2009). Selective KOR activation can also reinstate responding for alcohol (Funk *et al.*, 2014; Harshberger *et al.*, 2016), cocaine (Valdez *et al.*, 2007; Beardsley *et al.*, 2010), and nicotine (Grella *et al.*, 2014), likely through activation of brain stress responses. However, a recent preclinical evaluation of nalfurafine, a clinically approved KOR agonist for antipruritus, or itch relief, reported a promising

reduction in alcohol consumption with no significant effect on anxiety-like behavior, anhedonia, or dysphoria (Zhou & Kreek, 2019).

KOR blockade has mixed effects on drug-associated behaviors. KOR antagonists can attenuate or block stress-induced reinstatement of nicotine- and alcohol-seeking (Schank *et al.*, 2012; Funk *et al.*, 2014; Grella *et al.*, 2014; Nygard *et al.*, 2016), effects that are thought to be mediated by prevention of stress-induced increases in dynorphin release and subsequent reduction in dopamine release (Grella *et al.*, 2014). However, norBNI, a long-acting KOR antagonist, has also been shown to either increase (Mitchell *et al.*, 2005; Anderson *et al.*, 2013; Morales *et al.*, 2014), decrease (Walker & Koob, 2008; Walker *et al.*, 2011), or have no effect (Walker *et al.*, 2011; Walker *et al.*, 2012) on alcohol reward and reinforcement in rats. Some of these discrepancies may relate to dependent versus non-dependent animals, with enhanced KOR sensitivity during states of dependence (Walker & Koob, 2008; Walker *et al.*, 2012).

Nicotine reinforcement, in contrast, is likely differentially regulated by KORs, as KOR activity has been linked to enhanced nicotine reward following a stressor (i.e., forced swim stress) or KOR agonist administration (Smith *et al.*, 2012), as well as greater nicotine-induced aversion (Ward *et al.*, 2018). The long-acting KOR antagonists norBNI, GNTI, and JDTic have no effect on nicotine intake or CPP (Jackson *et al.*, 2010; Liu & Jernigan, 2011). Other nicotine-associated behaviors, however, may be mediated by KOR activity, as JDTic, norBNI, and the short-acting antagonist LY2456302, can attenuate both somatic and affective symptoms of nicotine withdrawal (Jackson *et al.*, 2010; Jackson *et al.*, 2015).

Although the modulatory role of KORs in behavioral or neurochemical responses to concurrent nicotine and alcohol is not well established, our lab demonstrated recently that systemic norBNI pretreatment enhances acquisition of intravenous Nic+EtOH self-administration in adult male rats, without influencing females or adolescent males (Lárraga *et al.*, 2017).

KORs and mood-related behaviors

Mood-associated behaviors and psychiatric disorders have also been linked to the dynorphin/KOR system. KOR agonists can induce negative affective behaviors, including anxiety, aversion, and dysphoria (Mague *et al.*, 2003; Todtenkopf *et al.*, 2004; Carlezon *et al.*, 2006; Narita *et al.*, 2006; Land *et al.*, 2009; Smith *et al.*, 2012; Gillett *et al.*, 2013; Van't Veer & Carlezon, 2013). As one would expect, selective KOR antagonists, including norBNI and JDTic, have anxiolytic and antidepressant effects (Mague *et al.*, 2003; Beardsley *et al.*, 2005; Knoll *et al.*, 2007; Reindl *et al.*, 2008; Carr *et al.*, 2010; Knoll *et al.*, 2011; Rorick-Kehn *et al.*, 2014).

However, these findings are not without discrepancies, as anxiolytic and anti-depressant effects of KOR activation have been reported (Privette & Terrian, 1995; Braida *et al.*, 2009; Alexeeva *et al.*, 2012; Harden *et al.*, 2012). These seemingly paradoxical effects, as reviewed below, are likely the result of developmental differences in KOR function, although sex differences may contribute too.

Sex differences in KOR function

Substantial evidence shows sex differences in KOR function and response to receptor activation (Chartoff & Mavrikaki, 2015). Both human women and female rodents are less sensitive than males to the antinociceptive and aversive effects of KOR agonists (Barrett *et al.*, 2002; Stoffel *et al.*, 2005; Wang *et al.*, 2011; Russell *et al.*, 2014). For example, U50,488 dose-dependently increases intracranial self-stimulation thresholds, a measure of depressive-like behavior, in adult male, but not female, rats (Russell *et al.*, 2014). Female C57Bl/6J and California mice also do not display a reduction in forced swim test immobility following KOR blockade, as in males (Laman-Maharg *et al.*, 2018).

An obvious potential mediator of these differences is ovarian hormones, as estrogens can increase receptor expression and are known regulators of dynorphin signaling (Gottsch *et al.*, 2009; Lawson *et al.*, 2010; Mostari *et al.*, 2013). However, there appears to be a divergence in the hormonal regulation of KOR function based on outcome measure. Whereas KOR-mediated analgesia is negatively regulated by estrogens, with estradiol-GRK2 interactions decreasing analgesic efficacy of U50,488 (Mogil *et al.*, 2003; Abraham *et al.*, 2018), the dysphoric and aversive effects of KOR activation are not altered by ovariectomy or stage of estrus cycle (Russell *et al.*, 2014; Abraham *et al.*, 2018). Mechanisms mediating females' decreased sensitivity to aversion and dysphoria following KOR activation are not currently known, but may be independent of dopamine neurotransmission (Abraham *et al.*, 2018).

KOR-mediated modulation of alcohol responses also differs based on sex, although data are limited. Female mice appear to be less sensitive than male mice to KOR agonist-induced decreases in binge-like alcohol consumption (Van't Veer *et al.*,

2016; Zhou *et al.*, 2017). Whereas receptor blockade with norBNI increased oral ethanol intake in males, it decreased intake in adult female rats (Morales *et al.*, 2014). Methodological differences, such as duration of exposure, route of administration, and group versus single housing, may explain these discrepancies, but these studies highlight the importance of including females in preclinical research focusing on the role of KOR signaling in mood- and drug-associated behaviors.

Adolescent maturation of KOR function

Although KORs appear early in ontogeny, recent evidence suggests that functional maturation continues through adolescence. Coupled with the profound maturation of monoamine systems, functional shifts in KOR function have important implications for mood- and drug-associated behaviors during this developmental period. KOR mRNA is detected in the rodent brain as early as gestational day (G) 13 (Georges *et al.*, 1998), but density of KOR binding differs regionally across early postnatal development. Adult levels of KOR binding are typically reached prior to adolescence, although thalamic binding sites gradually increase until P38 (Kornblum *et al.*, 1987). Early electrophysiological studies suggested that KOR stimulation can inhibit electrically evoked dopamine release in the striatum at G17, and KORs are therefore functionally active (De Vries *et al.*, 1990).

More recent work suggests that the KOR undergoes an important functional maturation during adolescence. Whereas activation of KORs in preweanling rats increases locomotor activity (Duke *et al.*, 1997; McDougall *et al.*, 1997), it reduces locomotor activity in adults (Duke *et al.*, 1997). Furthermore, while preweanling (P10 or

P17) and adult rats develop significant place preference to morphine that is attenuated by U50,488 pretreatment, adolescent (P35) rats do not develop morphine place preference and do not exhibit U50,488-induced changes in locomotor activity (Bolanos *et al.*, 1996). This may be due to an adolescent-specific decrease in functional sensitivity of the KOR. The aversive effects of KOR activation, measured by conditioned taste aversion (CTA), are attenuated in adolescent compared to adult rats (Anderson *et al.*, 2014). However, this age difference may depend on stress, as non-water deprived adolescents developed significant CTA to higher doses of the KOR agonist U62,066 (0.3-0.5 mg/kg; Anderson *et al.*, 2013).

A small subset of literature reports unexpected decreases in anxiety- and depressive-like behavior after KOR agonist treatment (Privette & Terrian, 1995; Braida *et al.*, 2009; Alexeeva *et al.*, 2012). Of note, these studies do not include older adult males for comparison, or list age of experimental animals. However, the reported weights for these “adult” males was ~200 g, which corresponds to adolescent animals, and provides further support for a functional maturation of KORs prior to adulthood.

KOR sensitivity may also be age-dependently altered by semi-chronic or chronic drug exposure, although this varies depending on the behavioral paradigm used. Whereas drug naïve adolescent and adult rats display comparable U50,488-induced increases in anxiety-like behavior and decreases in NAc dopamine levels, chronic nicotine treatment enhances KOR-mediated reductions in NAc dopamine in adults only (Tejeda *et al.*, 2012a). Similarly, nicotine-treated adolescents do not develop significant place aversion to U50,488, while adults do (Tejeda *et al.*, 2012a). In regards to alcohol, one group has found that adult male rats show increased oral ethanol intake after KOR

blockade, while adolescents show no change in intake (Morales *et al.*, 2014). Our lab has shown that Nic+EtOH is reinforcing in adolescent, but not adult, males and that a late-emerging inhibitory role of KORs strongly modulates Nic+EtOH reinforcement in males, while distinct mechanisms may exist in females (Lárraga *et al.*, 2017). Overall, these findings highlight the profound contribution of age and sex to KOR function.

V. Rationale for the dissertation

Mechanisms mediating high rates of nicotine and alcohol co-use and co-abuse are poorly understood. Adolescent exposure to nicotine can produce a strong "gateway" effect, increasing sensitivity to the rewarding properties of other drugs of abuse, including alcohol. Although normal maturational changes in monoamine systems have been linked to adolescent-specific increases in nicotine consumption, functional maturation of other neurotransmitter systems – particularly those that play a modulatory role over dopamine – likely provide an important contribution to these behaviors. The dynorphin/KOR system has significant regional and cellular overlap with dopamine neurocircuitry and is thus poised to exert critical modulation over mood, reward, and motivated behavior. A growing body of literature emphasizes key sex differences in KOR function, with females generally less sensitive to the effects of KOR activation, as well as a functional maturation during adolescence. However, the contribution of sex and age to KOR function has not been systematically evaluated in the context of behavioral and neurochemical responses to nicotine and alcohol combinations.

Therefore, this dissertation evaluates behavioral and neurochemical responses to nicotine and alcohol across development and between sex. These studies explore

how KORs modulate these behaviors, as well as examining their role in mediating anxiety- and depressive-like behaviors in adolescents and females. Specifically, this dissertation examines the behavioral, neurochemical, and functional effects of concurrent nicotine and alcohol in male rats during adolescence and adulthood (Chapter 2). I then show age- and sex-dependent changes in KOR-mediated modulation of acute behavioral responses to nicotine and alcohol, as well as regional differences in G-protein coupling efficacy that occur separately from developmental and/or sex differences in KOR expression (Chapter 3). In line with these studies, behavioral analyses demonstrate altered sensitivity to the anxiety- and depressive-like effects of KOR agonists based on age, sex, and behavioral measure (Chapter 4). As a whole, my work emphasizes the profound complexity of KOR signaling and highlights important maturational and sex-dependent changes in KOR function.

Chapter 2

Combined nicotine and ethanol age-dependently alter neural and behavioral responses in male rats

Introduction

Alcohol and nicotine, via tobacco or electronic cigarettes (e-cigarettes), are the two most commonly co-used substances. Concurrent use is associated with severe health consequences (Van Skike *et al.*, 2016), and it is estimated that the economic costs associated with alcohol and tobacco use in the U.S. totals almost 500 billion dollars annually (National Institute on Drug Abuse, 2015). The majority of individuals with AUD also smoke (Falk *et al.*, 2006), and dependent smokers are approximately ten times more likely than non-dependent smokers to have AUD (DiFranza & Guerrera, 1990).

Initiation of tobacco and alcohol use, either individually or concurrently, typically begins during adolescence. A recent assessment of 12th grade patterns of use from 1976-2010 has shown that, although overall drug use has declined, tobacco and alcohol co-use has increased (Daw *et al.*, 2013). Moreover, almost 90% of current adult smokers began smoking before the age of 18 (SAMHSA, 2011), and alcohol is typically first consumed before the age of 16 (Behrendt *et al.*, 2009). Early adolescent onset of smoking is associated with the greatest risk of excessive alcohol consumption and AUDs (Weitzman & Chen, 2005). E-cigarettes, which are now the most commonly used tobacco product among middle and high school students (Jamal *et al.*, 2017), are also

associated with increased alcohol use and misuse among adolescents (Hershberger & Cyders, 2017).

Adolescence is a transition period between childhood and adulthood conservatively estimated to last from 12 to 18 years of age in humans and postnatal days (P) 28-42 in rodents (Spear, 2000). It is highly conserved across mammalian species, and marked by major reorganization of limbic and cortical regions important for learning and memory, executive function, and reward processing (Spear, 2000; Yuan *et al.*, 2015). These changes are necessary for the transition to adult autonomy, but do leave the adolescent brain uniquely vulnerable to the detrimental effects of nicotine and alcohol. Consistent with prior work from our lab showing that brief, low-dose nicotine pretreatment during early adolescence (P28-31) enhances acquisition of alcohol self-administration (Dao *et al.*, 2011), more recent work demonstrates that adolescent male rats find concurrent intravenous self-administration of nicotine and alcohol significantly more reinforcing than adult male rats (Lárraga *et al.*, 2017). Adolescent nicotine exposure also produced long-lasting increases in oral alcohol consumption (Lárraga *et al.*, 2017), which is in line with epidemiological findings as mentioned above. This enhanced reinforcement of co-administered drugs, and the long-lasting increase in alcohol reward, is not evident in adult males or females of either age, and may be due to developmental differences in drug-induced neural activation in reward- and stress-related brain regions.

In the present study, I have examined the effects of combined nicotine and ethanol (Nic+EtOH) on locomotor and anxiety-like behaviors in adolescent and adult male rats. I hypothesized that adolescents would be more sensitive to the locomotor

stimulating and anxiolytic effects of Nic+EtOH than adults, and that drug-induced neuronal activity would be higher in regions mediating drug reward in adolescents. In behaviorally tested animals, I measured *cFos* mRNA expression, an immediate early gene widely used as a marker of neuronal activity (Flavell & Greenberg, 2008). I used this approach to examine regional changes in neuronal activity. I also adapted graph theory methods (Dwyer & Leslie, 2016) to construct and evaluate networks of coordinated gene expression (CGE) - analogous to functional connectivity - associated with acute exposure to Nic+EtOH in adult and adolescent male rats. I demonstrate that Nic+EtOH uniquely influences locomotor activity, anxiety-like behavior, regional neuronal activity, and functional connectivity in an age-specific manner. My findings add to the growing body of literature showing anomalous effects of combined versus individual exposure to nicotine and alcohol, and provide a potential mechanism for high rates of co-use of these drugs observed among teenagers and young adults via alterations in anxiety and functional neural circuitry.

Materials and Methods

Animals

Male Sprague Dawley rats were obtained from Charles River at P17 or P75 and juveniles were housed with a dam until weaning (P21). Females were not included as age and Nic+EtOH interactions were not observed in females in our prior study (Lárraga *et al.*, 2017). Weaned juveniles and adults (P76) were group housed in an AALAC-accredited vivarium on a 12-hour light-dark cycle with food and water available *ad libitum*. No more than one animal per litter per experimental group was used to avoid

potential litter effects (Lárraga *et al.*, 2017). All procedures were in compliance with NIH guidelines and approved by the Institutional Animal Care and Use Committee of the University of California, Irvine. All animals were handled for 2 min daily for three days prior to surgery and thereafter. All behavioral testing occurred during the light cycle.

Catheterization

Adolescent and adult rats underwent surgery at P28 and P86, respectively. Animals were anesthetized with Equithesin (0.035 mg/kg, i.p.), and were surgically implanted with a catheter into their right jugular vein (Belluzzi *et al.*, 2005). Rats were given three days to recover before drug exposure. Cannulae were flushed daily with heparinized saline solution to maintain catheter patency. Propofol (5 mg/kg, i.v.) was injected the day before behavioral testing; animals that did not display rapid (5-10 sec) anesthesia were excluded from further analysis.

Drug exposure and behavioral testing

Nicotine hydrogen tartrate was purchased from Glentham Life Sciences (London, UK), 100% ethanol was purchased from Sigma (St. Louis, MO), and propofol was purchased from Abbot Laboratories (Chicago, IL). Nicotine was dissolved in sterile saline and adjusted to pH 7.2-7.4. Ethanol was prepared at concentrations no greater than 20% (v/v). Adolescent (P32) and adult (P90) rats were allowed to habituate to the experimental room for 30 min in their home cages. Animals then received two injections of saline (1 ml/kg) or Nic+EtOH (2 x 15 μg/kg nicotine plus 2 x 2 mg/kg EtOH, i.v.) spaced 1 min apart. Immediately after treatment, animals were placed in a novel open-

field activity chamber (43.2 x 43.2 x 30.5 cm) connected to a common interface and computer (Med Associates Inc., St. Albans, VT) for 30 min. Ambulatory counts and time spent in the center of the open-field were recorded automatically; the center area was defined based on the average body size at each age. Immediately following behavioral testing, animals were decapitated, brains collected and flash frozen in 2-methylbutane at -20°C, and stored at -70°C until processing. The time chosen for tissue collection (i.e., 30 min after drug injection) corresponds to the peak of stimulus-induced *cFos* mRNA expression (Cullinan *et al.*, 1995).

Measurement of cFos *mRNA expression*

Twenty µm sections were cut and processed for *cFos in situ* hybridization. [^{35}S]-labeled UTP (Perkin Elmer, Boston, MA) was used to synthesize cRNA riboprobes for *cFos* in the sense and antisense orientation from a pGEM-3Z plasmid containing a 680bp fragment of *cFos* cDNA between T7 and SP6 promoter sites. *cFos* cDNA was kindly provided by Dr. Stanley J. Watson, University of Michigan. Tissue sections were pretreated with Proteinase K, acetylated, dehydrated through ascending concentrations of ethanol and then air dried. Sections were incubated for 16-18 hr at 60°C, with hybridization solution containing the [^{35}S]-labeled riboprobe (10^7 cpm/ml). Following hybridization, sections were incubated with RNase A and washed with descending concentrations of SSC before being dehydrated in ethanol and apposed in light-tight cassettes to Kodak BioMax film with [^{14}C] standards of known radioactivity for one day. Film was developed and rapidly fixed for quantitative analysis with a MicroComputer Imaging Device (MCID, Imaging Research, St. Catherine, Ontario, Canada), as

described earlier (Dwyer & Leslie, 2016). A calibration curve of optical density against radioligand concentration (dpm/mg tissue) was constructed using [^{14}C] brain paste standards. Optical densities in each region of interest were measured and values of radioactivity were calculated by interpolation from the calibration curve and averaged to give the value for a single region. Specific hybridization was calculated by subtracting values of radioactivity in sections hybridized with sense probe from those hybridized with antisense. Regional averages were obtained from readings of the left and right hemispheres from two comparable sections for every region. Brain areas in autoradiograms were identified with well-defined anatomical landmarks and with reference to adjacent brain sections processed for Nissl-staining.

Regions were selected *a priori*, based on their involvement in drug reward, anxiety-like behaviors, and previous work in our lab demonstrating age- and region-specific effects of nicotine exposure on neuronal activity (Cao *et al.*, 2007a; Yuan *et al.*, 2015). Areas where measurements for *cFos* expression were taken and included for network analysis are listed in Table 2.1 and include prefrontal cortex [ventral orbital (VO), lateral orbital (LO), cingulate (Cg1), prelimbic (prL), infralimbic (IL), dorsal peduncular (DP), and dorsal tenia tecta (DTT)], striatum [dorsomedial caudate putamen (dmCPu), dorsolateral caudate putamen (dlCPu), ventromedial caudate putamen (vmCPu), ventrolateral caudate putamen (vlCPu), nucleus accumbens shell (NAc-shell), and nucleus accumbens core (NAc-core)], bed nucleus of the stria terminalis [anterior (aBNST) and posterior (pBNST)], medial preoptic area (mPOA), hypothalamus [lateral (LH) and paraventricular (PVH)], amygdala [medial nucleus (MeA), central nucleus (CeA), and basolateral (BLA)], thalamus [paraventricular nucleus (PVT), centromedial

nucleus (CMT), and anteroventral nucleus (AVT)], substantia nigra (SN), ventral tegmental area [anterior (aVTA) and posterior (pVTA)], interpeduncular nucleus (IPN), superior colliculus (SC), periaqueductal gray (PAG), raphe nucleus [dorsal (DR) and median (MR)], and hippocampus [CA1, CA2, CA3, and dentate gyrus (DG)].

Table 2.1. Names and abbreviations of brain regions of interest

	Abbreviation	Full name		Abbreviation	Full name
Cortex	*VO*	Ventral orbital cortex	**Amygdala**	*MeA*	Medial amygdala
	LO	Lateral orbital cortex		*CeA*	Central amygdala
	Cg1	Cingulate cortex		*BLA*	Basolateral amygdala
	PrL	Prelimbic cortex	**Thalamus**	*PVT*	Paraventricular nucleus of the thalamus
	IL	Infralimbic cortex		*AVT*	Anteroventral nucleus of the thalamus
	DP	Dorsal peduncular cortex		*CMT*	Centromedial nucleus of the thalamus
	DTT	Dorsal tenia tecta	**Midbrain**	*aVTA*	Anterior ventral tegmental area
Striatum	*NAc-core*	Nucleus accumbens core		*pVTA*	Posterior ventral tegmental area
	NAc-shell	Nucleus accumbens shell		*SN*	Substantia nigra
	dmCPu	Dorsomedial caudate putamen		*IPN*	Interpeduncular nucleus
	dlCPu	Dorsolateral caudate putamen		*PAG*	Periaqueductal gray
	vmCPu	Ventromedial caudate putamen		*SC*	Superior colliculus
	vlCPu	Ventrolateral caudate putamen		*DR*	Dorsal raphe nucleus
Stress nuclei	*aBNST*	Anterior bed nucleus of the stria terminalis		*MR*	Median raphe nucleus
	pBNST	Posterior bed nucleus of the stria terminalis	**Hippocampus**	*CA1*	CA1
	PVH	Paraventricular nucleus of the hypothalamus		*CA2*	CA2
	LH	Lateral hypothalamus		*CA3*	CA3
	mPOA	Medial preoptic area		*DG*	Dentate gyrus

Analysis of coordinated gene expression (CGE)

In order to determine network-level CGE, I used a methodology developed by our lab (Dwyer & Leslie, 2016) and adapted from Rubinov and Sporns (2010). An adjacency matrix was constructed for each drug treatment and age group, yielding four matrices. Matrices were composed of undirected, weighted Pearson coefficients (r) from the intersubject correlation of *cFos* expression between each pair of brain regions analyzed (36×36 regions). Matrices were thresholded at $p<0.05$ and non-significant r-values were set to zero for visualization purposes. Visual maps of CGE were constructed by importing thresholded matrices into UCINET Netdraw (UCINET 6.0, Analytic Technologies, Lexington, KY). Analyzed brain regions were arranged relative to their anatomical location in a mid-sagittal section of the brain. Significant r-values denoting association between two brain regions (CGE, analogous to functional connectivity) were displayed as lines (edges) connecting two brain regions (nodes). The thickness of the line denotes the strength of the r-value, while color distinguishes r-values that were negative (red) or positive (black).

Properties of each functional network were characterized statistically using a number of measures from the open-source brain connectivity toolbox (Rubinov & Sporns, 2010; Matlab R2016b, MatWorks, Natick, MA). *(I) Modularity*. Modularity quantifies and separates networks into optimized community structure comprised of subcommunities with maximal within-group connections and minimal between-group connections. The modularity function (modularity_und.m) assigns each node to an individual community, and functional subcommunities are denoted by color in network visualizations. *(II) Degree*. The degree of a node represents the number of significant

correlations, or functional relationships, that a given node has with other nodes in the network. The undirected and unweighted degree function was used (degrees_und.m). *(III) Betweenness Centrality.* Betweenness centrality is a nodal property that reflects the amount of control a node has over the interactions of other nodes within the network, favoring nodes that join communities rather than simply exist within communities. It represents the fraction of shortest paths in the network passing through a given node, and was assessed using the weighted betweenness centrality function (betweenness_wei.m) and an inverse mapping of weighted matrices (i.e., mapping where tighter functional relationships are interpreted as shorter distances). *(IV) Hub analysis*. Hubs are nodes that are poised to contribute strongly to global network function. Nodes were designated as hubs based on two criteria: degree and betweenness centrality. If a node's degree or betweenness centrality value was at least two standard deviations from the network mean, it was denoted as a hub (Bassett *et al.*, 2008). Hubs are denoted in network visualizations by node size and solid color fill. *(V) Clustering Coefficient.* The clustering coefficient is a measure of functional segregation, or the ability for specialized processing to take place among highly interconnected groups of brain regions. It is equivalent to the fraction of node's neighbors that are neighbors of each other, and was assessed using the binary, undirected clustering coefficient function (clustering_coef.bu.m). *(VI) Global efficiency.* Global efficiency is a measure of functional integration, or how well a network is able to exchange information, and is the average inverse shortest path length in the network. The characteristic path length (charpath.m) function was used to assess global efficiency.

Data analyses

Behavioral data analysis: Data are expressed as mean + SEM. Locomotor and center time data were analyzed by two-way ANOVA for Age x Drug. All significant main or interaction effects were further analyzed by one-way ANOVA with Bonferroni-adjusted *post hoc* comparisons (GraphPad Prism 6.0, GraphPad Software, San Diego, CA). Outliers (≥2 SD from group mean) were removed from each cohort of animals separately before data were combined for analysis.

Regional mRNA expression: Data are expressed as mean + SEM and were analyzed by two-way ANOVA for Age x Treatment. All significant main or interaction effects were further analyzed by one-way ANOVA with Bonferroni-adjusted *post hoc* comparisons. All analyses were completed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA).

Network differences in CGE: Drug-treated adolescent and adult *cFos* mRNA CGE networks were compared with networks of their saline-treated counterparts to determine differences in functional relationships. While thresholded networks were used for visualization, all r-values were included for network comparisons so that significant differences between strong and weak networks could be evaluated. R-values for each interregional correlation were transformed to z scores in order to improve normality. Differences between treatment groups were determined by dividing the subtracted z scores by the standard error of the difference. Z difference scores were then converted to p-values, which were corrected using false discovery rate (Genovese *et al.*, 2002). A

moderate false discovery rate (q=0.10) was chosen to minimize Type II errors that may be increased when conservative thresholds are used in networks with low signal-to-noise ratios, while still providing correction against Type I errors. For visualization, the brain regions analyzed were arranged relative to their anatomical location in a mid-sagittal section of the brain. Significant changes in CGE between two brain regions as a result of drug treatment were displayed as lines (edges) connecting two brain regions (nodes). The color and style were used to distinguish between a gain (dash, black) or a loss (dash, red) of significant CGE.

Results

Nic+EtOH increases locomotor activity in adolescents, but not adults

I observed significant effects of age and Nic+EtOH exposure on locomotor activity (Figure 2.1A). Since overall ANOVA indicated significant main effects of Age ($F_{1,66}$=24.647, p<0.001) and Drug ($F_{1,66}$=18.993, p<0.001), as well as a significant Age*Drug interaction ($F_{1,66}$=13.042, p=0.001), data were split by age to analyze the effects of Nic+EtOH. Compared to saline-treated controls, Nic+EtOH increased locomotor activity in adolescents (p<0.001), but not adults. Furthermore, Nic+EtOH-treated adolescents had higher locomotor activity than Nic+EtOH-treated adults (p<0.001).

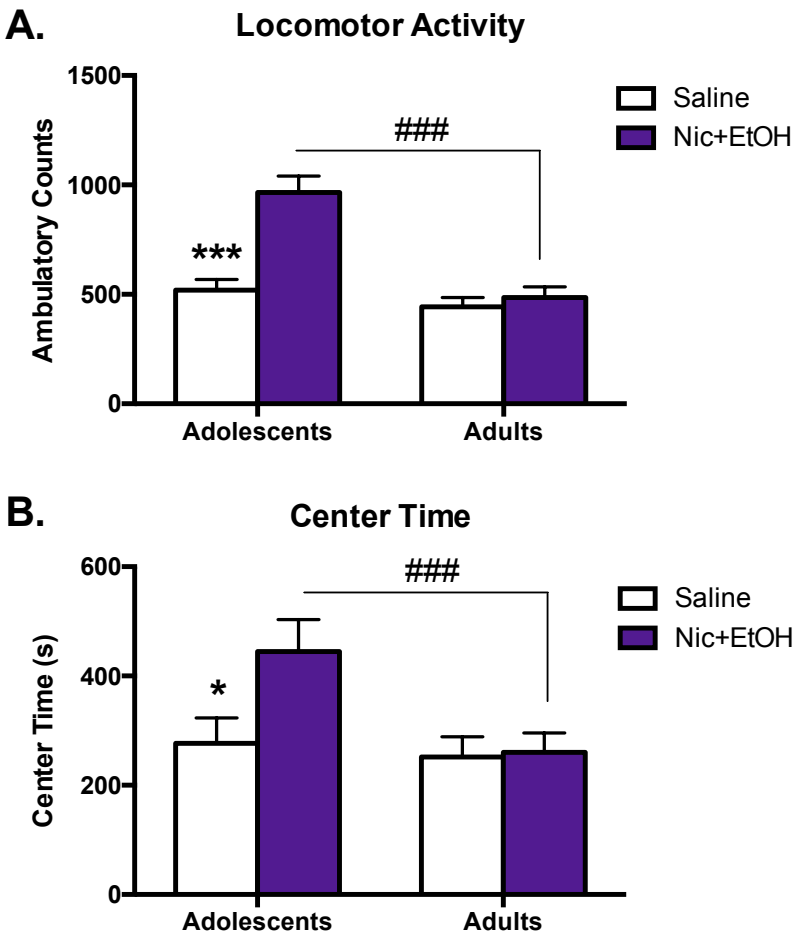

Figure 2.1. Nic+EtOH increases locomotor activity and center time in adolescent, but not adult, rats. (A) Total ambulatory counts and (B) time spent in the center zone of a novel open-field chamber. *, p<0.05; ***, p<0.001 vs. adults; ###, p<0.001 vs. Nic+EtOH. Data represent mean + SEM. n = 16-18/group.

Nic+EtOH is anxiolytic in adolescents, but not adults

In a measure of anxiety-like behavior, time spent in the center of an open-field, I found a significant main effect of Age ($F_{1,66}$=5.333, p=0.024) and a trend for a main effect of Drug ($F_{1,66}$=3.774, p=0.056). Data were split by age to examine my *a priori* hypothesis that Nic+EtOH would decrease anxiety-like behavior in adolescents. As hypothesized, Nic+EtOH increased time spent in the center zone in adolescents

(p=0.035), but not adults. Nic+EtOH-treated adolescents also spent more time in the center than Nic+EtOH-treated adults (p=0.012; Figure 2.1B).

Regional neuronal activity

To explore which brain regions may mediate age differences in the behavioral effects of Nic+EtOH, regional *cFos* mRNA expression was measured. There were significant main effects of Age in the aBNST ($F_{1,60}$=7.302, p=0.009), pBNST ($F_{1,67}$=4.089, p=0.047), mPOA ($F_{1,50}$=14.693, p<0.001), SN ($F_{1,63}$=7.436, p=0.008), IPN ($F_{1,60}$=5.746, p=0.020), MR ($F_{1,62}$=6.222, p=0.015), and CA2 ($F_{1,57}$=7.965, p=0.007), but no significant effects of Age or Age*Drug interactions. In all of these areas except the mPOA, neuronal activation was higher in adults than adolescents (Figure 2.2).

Significant main effects of Drug were found for NAc-core ($F_{1,65}$=6.312, p=0.014), PVT ($F_{1,64}$=6.970, p=0.010), AVT ($F_{1,64}$=6.254, p=0.015), CMT ($F_{1,66}$=4.311, p=0.042), MeA ($F_{1,64}$=12.157, p=0.001), CeA ($F_{1,52}$=4.367, p=0.042), BLA ($F_{1,66}$=7.636, p=0.007), PAG ($F_{1,56}$=10.458, p=0.002), DR ($F_{1,59}$=5.171, p=0.027), CA1 ($F_{1,57}$=12.982, p=0.001), and DG ($F_{1,57}$=4.597, p=0.036), but there were no significant effects of Age or Age*Drug interactions. Nic+EtOH decreased *cFos* mRNA levels in all of these regions, except the CeA where Nic+EtOH increased *cFos* (Figure 2.3).

Age and Nic+EtOH also interacted to influence *cFos* expression in some regions (Figure 2.4). Significant Age*Drug interactions were found in the pVTA ($F_{1,57}$=5.419, p=0.023), NAc-shell ($F_{1,68}$=6.111, p=0.016), LO ($F_{1,63}$=7.579, p=0.008), CA3 ($F_{1,52}$=7.100, p=0.010) and aVTA ($F_{1,55}$=5.911, p=0.018). In all of these regions, except pVTA, Nic+EtOH selectively decreased *cFos* expression in adults with no significant

effect in adolescents. In the pVTA, the drug combination selectively increased *cFos* expression in adults (p=0.047) with no effect in adolescents.

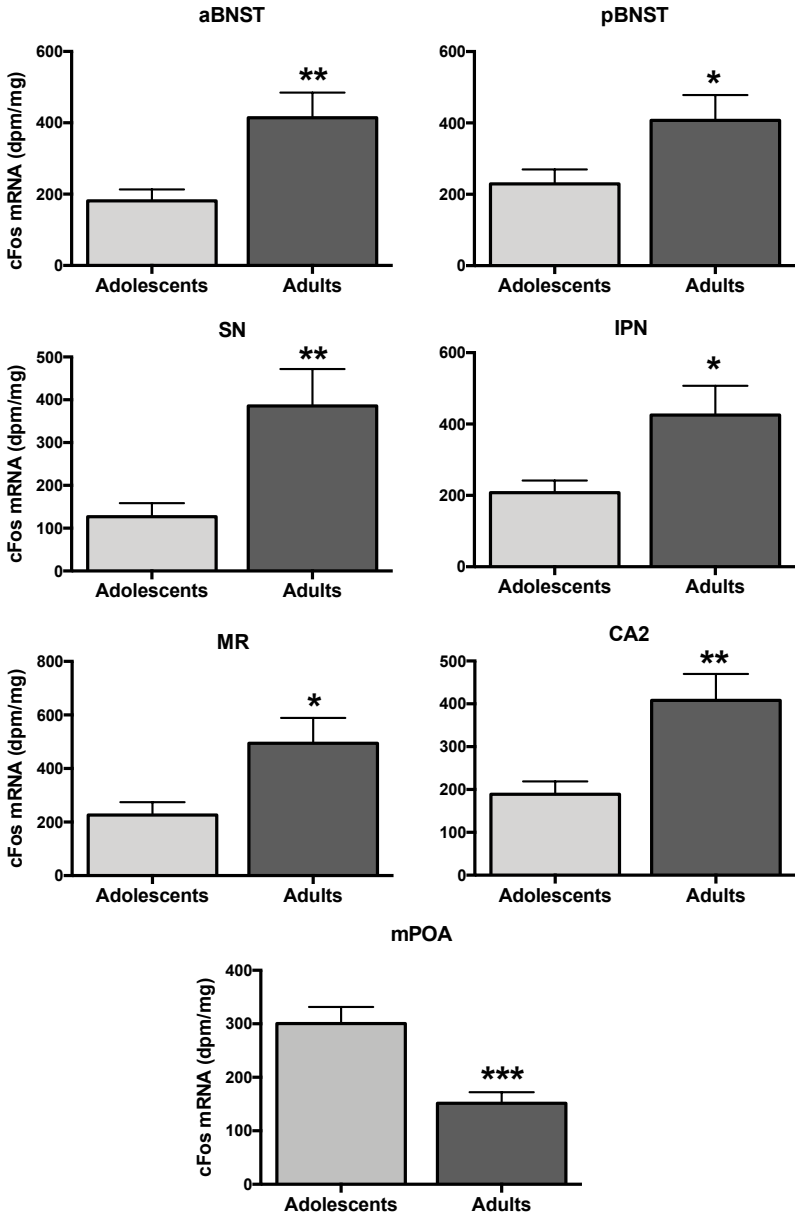

Figure 2.2. Age differences in regional *cFos* mRNA expression. Adults have higher *cFos* mRNA expression in extended amygdala, midbrain, and hippocampal areas, but lower *cFos* expression in the mPOA compared to adolescents, independent of drug exposure. *, p<0.05, **, p<0.01, ***, p<0.001 vs. adolescents. Data represent mean + SEM and are collapsed across drug group. n = 25-39/age.

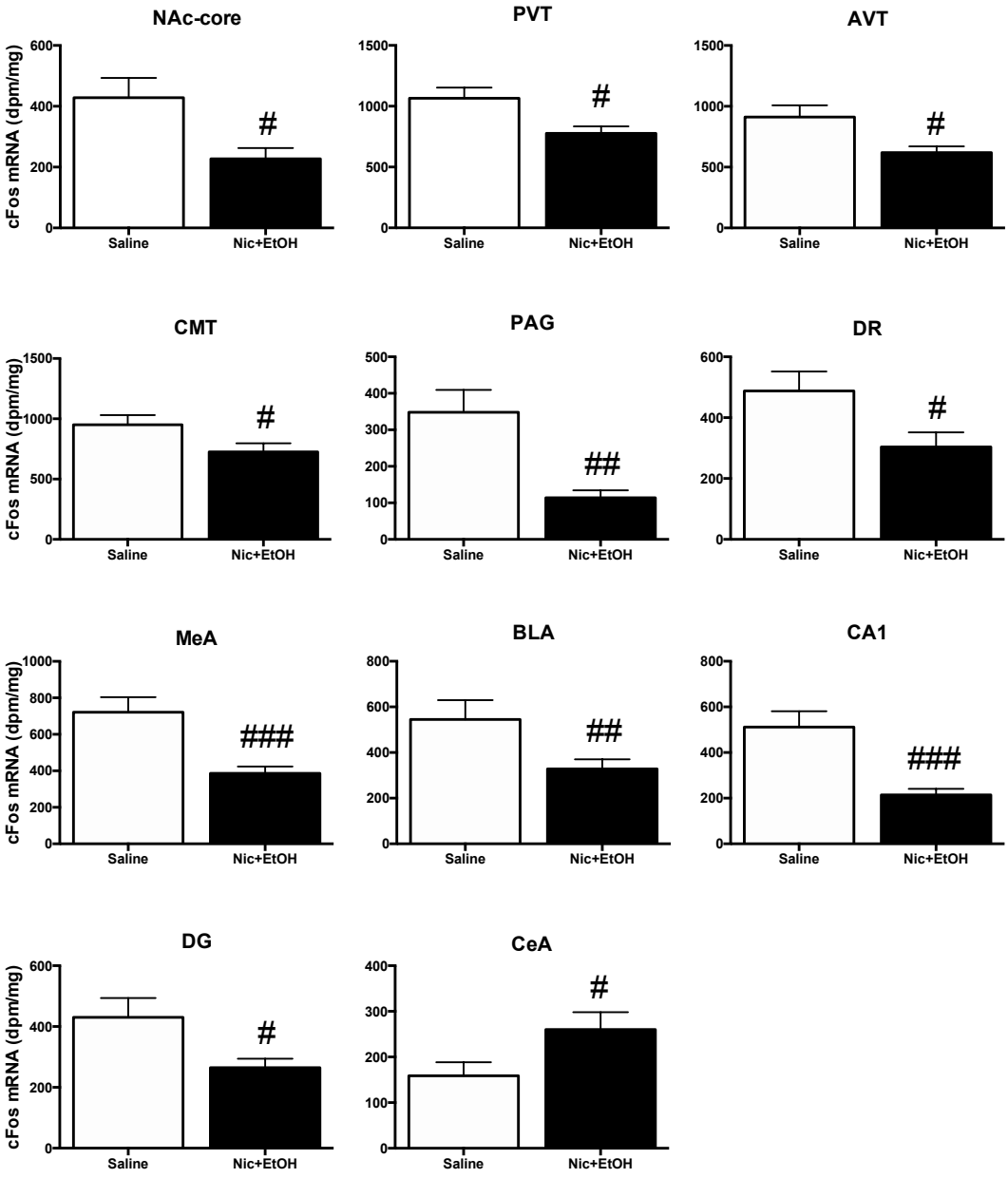

Figure 2.3. Drug differences in regional *cFos* mRNA expression. Nic+EtOH had region specific effects on *cFos* mRNA expression, independent of age. Drug-induced differences in regional *cFos* mRNA expression. #, p<0.05, ##, p<0.01, ###, p<0.001 vs. saline. Data represent mean + SEM and are collapsed across age. n = 27-38/drug.

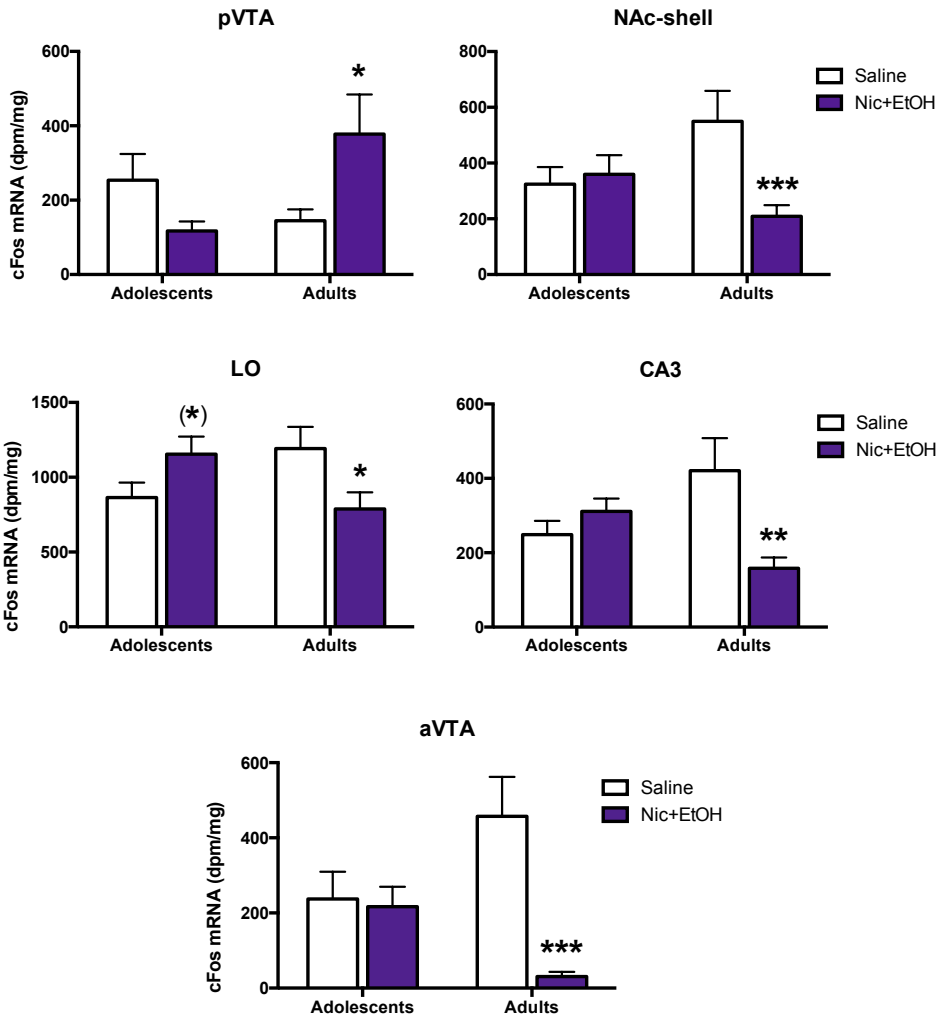

Figure 2.4. Age-specific effects of Nic+EtOH on regional cFos mRNA expression. (A) Nic+EtOH increases regional neuronal activity in the pVTA of adults, but not adolescents. In the NAc-shell (B), LO (C), CA (D), and aVTA (E), Nic+EtOH decreased *cFos* mRNA expression in adults compared to saline. *, p<0.05; **, p<0.01; ***, p<0.001 vs. saline. Data represent mean + SEM. n = 12-20/group.

Age differences in baseline and Nic+EtOH-mediated CGE networks

To examine baseline developmental differences in functional connectivity, networks of coordinated *cFos* gene expression were created and network properties were assessed for saline-treated adolescents and adults (Figure 2.5). Adolescents had unique community structure, with each community representing highly interconnected and segregated functional subgroups, as well as unique network hubs poised to exert

greater influence over global network function (i.e., DP, SC, and PAG) compared to adults (i.e., DP and dlCPu; Table 2.3). Adolescents (Figure 2.5A) had significantly fewer functional relationships than adults (p=0.005; Figure 2.5B).

Table 2.2. Network hub analysis.

Drug	Age	Region	k #SDs	Bc #SDs
Saline	**P32**	PAG		2.72
		SC		2.55
		DP		2.43
	P90	DP		3.17
		dlCPu		2.70
Nic+EtOH	**P32**	SC		3.47
		DP		2.48
		Cg1	2.04	2.04
		VO	2.04	
	P90	VO		3.11
		DP		2.74
		BLA		2.54
		CA2		2.16

Nodes are designated as network hubs if its degree (k) and/or betweenness centrality (Bc) value was at least two standard deviations (SDs) from the network mean.

As with baseline networks, Nic+EtOH-mediated networks displayed unique community structure and network hubs in adolescents compared to adults. In Nic+EtOH-treated adolescents (Figure 2.5C), network hubs included the Cg1, VO, and DP, while adults (Figure 2.5D) network hubs included VO, DP, hippocampal CA2, and BLA (Table 2.2).

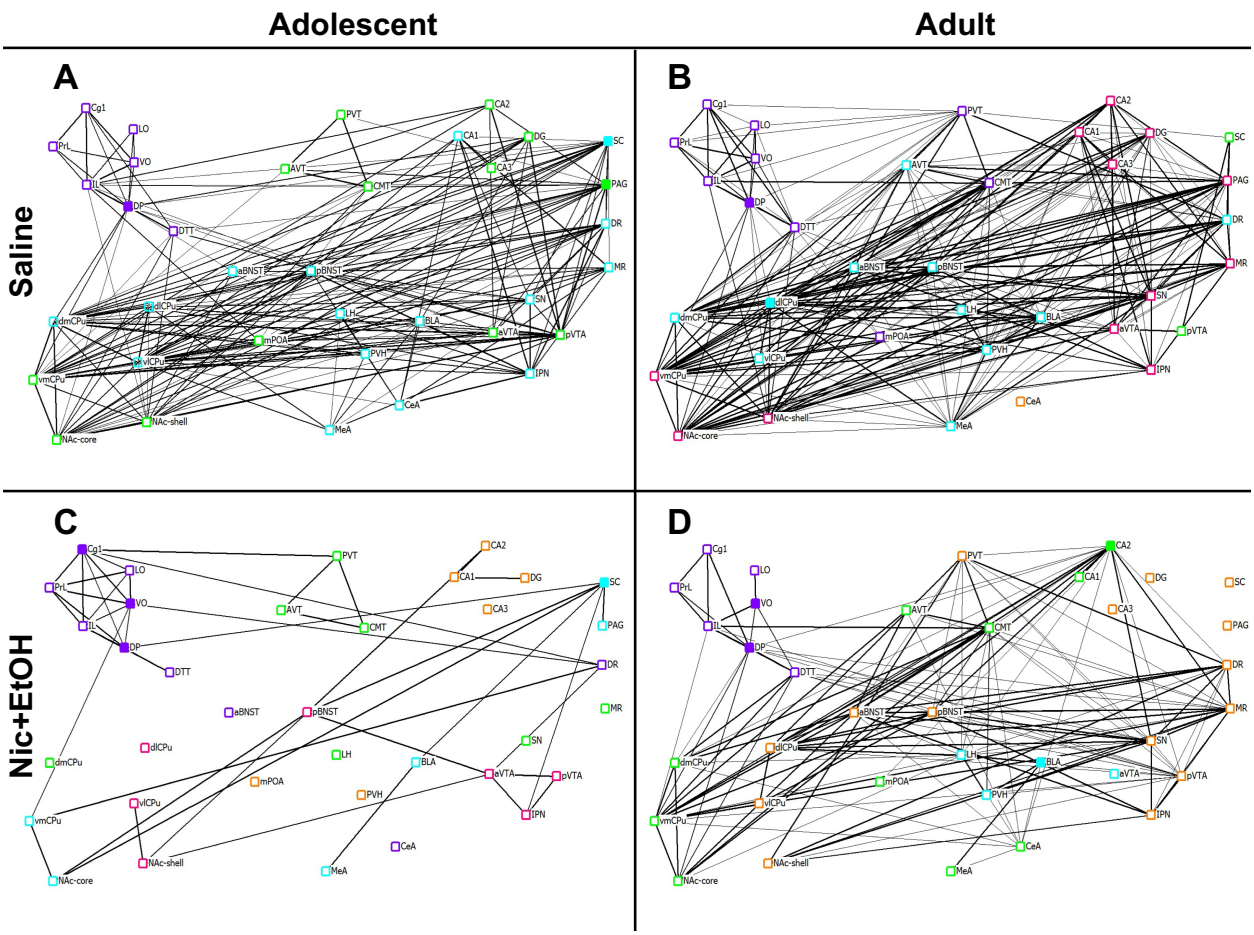

Figure 2.5. Baseline and Nic+EtOH-mediated functional networks. Networks of correlated *cFos* mRNA expression in saline-treated (A, B) or Nic+EtOH-treated (C, D) adolescents and adults. Each node represents a brain region presented in pseudoanatomical space. Significant r-values ($p<0.05$) denoting association between two brain regions (CGE, analogous to functional connectivity) were displayed as lines (edges) connecting two brain regions (nodes). The thickness of the line denotes the strength of the r-value, while color distinguishes between r-values that were negative (red) and positive (black). Functional subcommunities are denoted by node color. Network hubs, designated based on degree and betweenness centrality, are denoted by node size and solid color fill.

Age-dependent effects of Nic+EtOH on functional connectivity

Drug-induced CGE changes were assessed by comparing Nic+EtOH networks to saline networks at each age (Figure 2.6). The number of functional relationships was significantly decreased by Nic+EtOH in adolescents ($p<0.001$), but not adults ($p=0.09$). However, drug treatment did significantly and uniquely alter functional connectivity at both ages. In adolescents, the dominant effect of Nic+EtOH was a significant loss of

positive CGE between midbrain and striatal nuclei. Specifically, positive CGE was lost between pVTA and striatal nuclei (i.e., dmCPu, vmCPu, vlCPu, and NAc-core), as well as DG. Positive CGE between SN and vlCPU, as well as from the IPN to DR, MR, and CA1 was also lost as a result of adolescent Nic+EtOH exposure, as was connectivity between MR and CA1. There were no significant gains of functional interactions resulting from adolescent Nic+EtOH exposure.

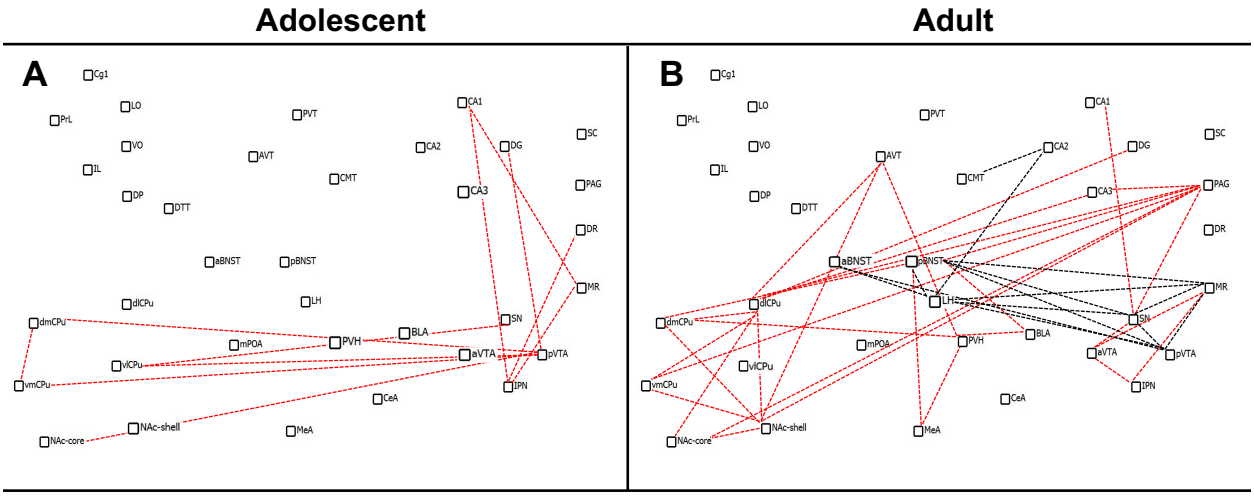

Figure 2.6. Nic+EtOH-induced changes in functional networks. Difference maps visually represent significant differences (corrected by false discovery rate, q=0.10) between baseline saline networks and Nic+EtOH networks in (A) adolescents and (B) adults. Each node represents a brain region presented in pseudoanatomical space. Lines indicate functional relationships between nodes that are significantly changed by Nic+EtOH treatment, where dashed red lines indicate a loss of positive *cFos* CGE and dashed black lines indicate a gain of positive *cFos* CGE.

In adults, drug-induced connectivity changes were more extensive, with both gains and losses of positive CGE (Figure 2.6B). Nic+EtOH produced a profound, significant loss of positive CGE within striatal nuclei, as well as some loss in connectivity within midbrain nuclei (i.e., aVTA with SN, IPN, and MR; IPN with MR). Numerous functional connections with the PAG (i.e., with hippocampal CA3, SN, NAc-core, NAc-shell, dmCPu, dlVPu, and vmCPu) were also lost after adult Nic+EtOH exposure. There

was a significant loss of positive CGE between hypothalamic (i.e., PVH) and amygdalar regions (i.e., MeA and BLA), as well as between the AVT and NAc-shell, dlCPu, and PVH. Nic+EtOH exposure also resulted in multiple gains of positive CGE, primarily between midbrain and extended amygdala nuclei. Specifically, Nic+EtOH led to a gain of connectivity between the pVTA and the aBNST and pBNST, as well as between pBNST and both SN and MR. Connections between the LH and pVTA, aBNST, and hippocampal CA2 were also gained after Nic+EtOH exposure in adults.

Discussion

Consistent with our prior behavioral observation that the combination of nicotine and ethanol is reinforcing in adolescent, but not adult, male rats (Lárraga *et al.*, 2017), I now show that Nic+EtOH treatment has unique, age-specific effects on both behavior and neuronal function. This drug combination increases locomotion and decreases anxiety in adolescent, but not adult, males. In contrast, neuronal activity, as measured by *cFos* mRNA expression, was selectively altered by drug exposure in a subset of regions in adult brain, being increased in the adult pVTA, but decreased in the aVTA, NAc-shell, LO, and hippocampal CA3. Coordinated gene expression was disrupted by Nic+EtOH in adolescents, as indicated by a large decrease in the number of functional connections compared to baseline networks. However, the organization of functional networks in adults was more profoundly altered by Nic+EtOH, with significant bidirectional changes in CGE within and between striatal, extended amygdala, and midbrain nuclei. My findings suggest that even brief, low-dose exposure to Nic+EtOH

can produce marked changes in behavior and neuronal function that differ widely based on age.

Age-dependent effects of Nic+EtOH on locomotor and anxiety-like behavior

In this study I have shown that acute Nic+EtOH treatment induces selective locomotor and anxiety-like behavior in adolescent male rats. Nic+EtOH increases ambulatory activity in adolescents, but not adults. Although prior studies have shown that the addition of ethanol reverses nicotine-induced locomotion increases (Abreu-Villaça *et al.*, 2008; Gulick & Gould, 2009), my data are in line with other reports highlighting adolescents' differential responses to nicotine or ethanol individually. For example, acute nicotine increases locomotor activity in adolescents, but decreases it in adults (Cao *et al.*, 2010), and adolescents display lower sensitivity to alcohol's sedative and motor-impairing effects (White *et al.*, 2002).

I have also shown that Nic+EtOH increases the amount of time that adolescents, but not adults, spend in the center of an open-field, indicating an anxiolytic effect. This Nic+EtOH effect on anxiety-like behavior is unlikely to be driven by drug-induced increases in ambulatory activity, as these behaviors are not correlated ($r=0.386$, $p=0.113$; data not shown), and we have shown previously that nicotine-induced alterations in center time are unrelated to locomotor activity (Cao *et al.*, 2010). Indeed, prior research from our lab and others has shown that nicotine alone is similarly anxiolytic in adolescents (Elliott *et al.*, 2004; Cao *et al.*, 2010). In contrast, recent work suggests that adolescents require higher doses of ethanol to experience its anxiolytic effects (Sakharkar *et al.*, 2014). However, my data adds to the growing body of

literature highlighting the unique effects of combined nicotine and ethanol on both behavior and neurochemistry (Cross *et al.*, 2017), and further underlines the importance of including age comparisons in experimental studies. Further studies should include other measures of anxiety-like behavior, such as the elevated plus maze or light-dark box, to more fully assess Nic+EtOH's effect on anxiety across development. However, the age-specific anxiolytic effect of Nic+EtOH that I observed here may suggest that concurrent nicotine and alcohol use by teenage and young adult males is due, in part, to their combined anxiety-reducing effects. This is in line with epidemiological data suggesting that greater anxiety among teenagers is associated with greater alcohol or concurrent nicotine and alcohol use (Comeau *et al.*, 2001; Malmberg *et al.*, 2010).

Regional neuronal activity in response to acute Nic+EtOH

I have also assessed how concurrent nicotine and ethanol treatment affects expression of *cFos* mRNA, an immediate early gene widely used as a marker of neuronal activity, in both adolescents and adults (Flavell & Greenberg, 2008). Adolescents had lower baseline *cFos* mRNA expression in the BNST, SN, IPN, MR, and CA2, but higher *cFos* expression in the mPOA, independent of drug treatment. Furthermore, neuronal activity was altered by Nic+EtOH at both ages in the NAc-core, thalamus, amygdala, PAG, DR, CA1, and CA2.

Interactions between age and drug treatment on regional *cFos* induction were limited, with Nic+EtOH decreasing neuronal activity in the NAc-shell, aVTA, LO, and CA3 of adults, but not adolescents. These adult-specific alterations in neuronal activity contrast with prior studies of nicotine treatment alone, in which *cFos* expression has

been shown to be increased in adolescents more than adults in reward-related brain areas, including the NAc, BNST, amygdala, and VTA (Shram *et al.*, 2007; Dao *et al.*, 2011). Acute ethanol, on the other hand, induces similar regional *cFos* expression in both adolescents and adults (Faria *et al.*, 2008), and has been shown to attenuate nicotine's effects on IEG induction (Bachtell & Ryabinin, 2001).

Of note, however, Nic+EtOH increased *cFos* mRNA expression in the adult pVTA, a region highly implicated in drug reinforcement (Sanchez-Catalan *et al.*, 2014), that may act as a key locus for nicotine and alcohol's effects. Indeed, ethanol and nicotine are individually self-administered into the pVTA, but not aVTA (Rodd-Henricks *et al.*, 2000; Rodd *et al.*, 2005; Ikemoto *et al.*, 2006; Ding *et al.*, 2015), and nicotine-induced dopamine cell firing is greater in the pVTA than the aVTA (Li *et al.*, 2011; Zhao-Shea *et al.*, 2011). This distinction is thought to be mediated by differential projection targets (i.e., NAc-shell versus NAc-core, respectively; Ikemoto *et al.*, 1997; Ikemoto, 2007), sensitivity to ethanol-induced dopamine neuron activity (Guan *et al.*, 2012), and expression of α4, α6, and β4 nicotinic acetylcholine receptors in the pVTA (Hendrickson *et al.*, 2010). My finding that *cFos* expression in adults was increased by low dose Nic+EtOH in the pVTA, but decreased in its major target region, the NAc-shell, may suggest that drug-induced activation of lateral hypothalamic dynorphin inputs to the pVTA (Muschamp *et al.*, 2014; Baimel *et al.*, 2017) inhibit dopamine projections to medium spiny neurons in the striatum, blocking the reinforcing effects of Nic+EtOH (Lárraga *et al.*, 2017). Future studies are needed to test this hypothesis.

Functional connectivity is age-dependently influenced by Nic+EtOH exposure

Discrete brain regions can coordinate activity with one another to form functional networks, or functional connectivity, even in the absence of direct anatomical connections (Sporns & Honey, 2006; Rubinov & Sporns, 2010). Functional connectivity, therefore, represents deviation from statistical independence between brain regions (Sporns, 2002). In this data-driven approach, graph theoretical methods, typically used to connect large data sets, have been adapted and applied to human imaging studies to identify multiple functional networks (e.g., the default mode network and the executive network) in the human brain, and statistically determine how development and drug exposure may affect functional connectivity. Indeed, functional connectivity changes across adolescence, shifting from local to distributed interactions that are important for mature executive and cognitive control (Crews *et al.*, 2007; Fair *et al.*, 2009; Hwang *et al.*, 2010; Satterthwaite *et al.*, 2013). Furthermore, mature inhibitory control is associated with improved long-range functional connectivity between subcortical and frontal regions, and concomitant decreases in short-range, within-region connectivity in the frontal and parietal cortices (Hwang *et al.*, 2010).

Methods in human imaging (e.g., fMRI, PET, EEG) that are typically used for functional network analysis are difficult to use in rodents (Liang *et al.*, 2013). Instead, measurement of immediate early gene expression (Pevzner *et al.*, 2012; Dwyer & Leslie, 2016) or cytochrome oxidase activity (Conejo *et al.*, 2010; González-Pardo *et al.*, 2012) allows for high spatial resolution readouts of neuronal activity and functional connectivity. Although studies are limited, these have provided evidence for

developmental- and drug-related alterations in functional connectivity in the rodent brain.

To assess developmental changes in functional connectivity, as well as the effects of acute concurrent nicotine and ethanol treatment, I used *cFos* as the readout of neuronal activity. In comparing baseline networks of adolescents and adults, I found unique community structures and hubs, as well as significantly fewer functional relationships, in adolescents compared to adults. This was unexpected given previous work from our lab (Dwyer & Leslie, 2016) and human imaging data (Stevens *et al.*, 2009) suggesting that functional connectivity shifts from local, highly coordinated networks in adolescence to more concentrated and efficient networks in adulthood. However, adults did have significantly higher clustering coefficients ($p<0.001$), a measure of functional segregation, or the ability for specialized processing to take place among interconnected groups of brain regions (Rubinov & Sporns, 2010). Global efficiency, a measure of functional integration, was not significantly different between adolescents and adults. Together, my findings demonstrate developmental shifts in functional network composition and properties.

Although there were few age and drug interactions in *cFos* mRNA expression in discrete brain areas, I observed robust, age-dependent alterations in functional networks after acute Nic+EtOH exposure. The number of functional relationships was decreased by Nic+EtOH in adolescents, but not adults, although drug-induced changes in connectivity were more extensive in adults. In adolescents, the predominant effect of drug exposure was a loss of positive connectivity between the pVTA and striatal nuclei, and between IPN/MR and hippocampus. Adults, on the other hand, exhibited a

considerable loss of positive intra-striatal and intra-midbrain connectivity, as well as a loss of functional relationships between the PAG and striatal nuclei, and between hypothalamic and extended amygdala nuclei. There were also multiple gains of positive CGE, primarily between the BNST and pVTA, SN, or MR resulting from Nic+EtOH exposure in adults. Of note, functional relationships with the pVTA were influenced by drug exposure at both ages, but in opposite directions. The pVTA has been implicated in the behavioral and neurochemical effects of nicotine and alcohol (Sanchez-Catalan *et al.*, 2014), but little is known about pVTA function or its interactions with other limbic circuitry during adolescence. However, my data suggest that functional networks involving the pVTA are differentially recruited by Nic+EtOH to modulate reinforcement and anxiety-like behavior in adolescence versus adulthood. Protective mechanisms, such as BNST glutamate inputs to non-dopaminergic neurons in the VTA that drive aversion and block reward (Jennings *et al.*, 2013; Vranjkovic *et al.*, 2017) may be recruited by Nic+EtOH exposure in adults to increase pVTA cFos expression and inhibit reinforcement (Lárraga *et al.*, 2017), while they are not recruited in adolescence. Similarly, a dynorphin projection from LH to dopamine cells in pVTA may be recruited in adults to inhibit Nic+EtOH-induced reinforcement via a KOR-dependent mechanism. Although future work with cell- and site-specific targeting is needed to test these hypotheses, my data highlight the complex maturation of limbic circuitry involved in regulating the acute effects of Nic+EtOH.

These findings complement recent human imaging data reporting greater and more diffuse hypoconnectivity in resting state functional networks of individuals who smoke and drink compared to smokers alone or drinkers alone (Vergara *et al.*, 2017).

The age-specific alterations in functional connectivity, and the relative insensitivity of adolescents to Nic+EtOH-induced neuronal activation in the pVTA, reported here, again highlight the importance of including age comparisons in preclinical research on nicotine and alcohol, as well as including age of initiation in clinical and epidemiological studies.

Conclusion

In the present study, I have demonstrated unique behavioral and neuronal responses to acute Nic+EtOH treatment in adolescent and adult male rats. Analysis of drug-induced *cFos* expression in individual brain areas showed that the pVTA, a critical regulator of drug reward, is selectively activated by Nic+EtOH in adults, whereas activity in its target region, the NAc-shell, was decreased. Drug-induced alterations in CGE are more extensive in adults compared to adolescents, and may act to inhibit adolescent behavioral responses to Nic+EtOH. Both regional *cFos* expression and network analysis suggest that there may be an ongoing maturation of the pVTA during adolescence that allows increased sensitivity to Nic+EtOH's reinforcing, hyperlocomotor, and anxiolytic effects.

My data add to the growing body of literature illustrating the unique effects of concurrent nicotine and alcohol (Cross *et al.*, 2017). Although it has been well established that developmental exposure to nicotine or alcohol can produce long-lasting alterations in reward and motivated behavior, few preclinical studies have examined the neurobiological consequences of this drug combination during adolescence. My research shows that even low doses of Nic+EtOH can produce marked alterations in brain and behavior that are distinct from what is observed in adults. Whether these

changes are long-lasting or predictive of subsequent maladaptive behaviors requires further study. However, my work provides a potential mechanism for the high rates of co-use of nicotine and alcohol by teenagers and young adults. That is, nicotine and alcohol combinations recruit functional networks in adolescence that are unique from protective, inhibitory networks recruited in the mature, adult brain. Given recent increases in e-cigarette use among teens and its association with alcohol consumption, these findings support greater emphasis of future research, as well as policy and public health strategies, on concurrent nicotine and alcohol intake.

Chapter 3

Developmental and sex differences in kappa opioid receptor-mediated regulation of nicotine and alcohol responses

Introduction

Alcohol and nicotine are the most commonly co-used substances, and their use typically begins during adolescence. Adolescence is a critical developmental period marked by major reorganization of brain regions critical for executive function, learning and memory, and reward processing (Spear, 2000; Casey *et al.*, 2008). As a result, the adolescent brain is highly sensitive to exogenous influences, such as nicotine (Yuan *et al.*, 2015). The majority of current adult smokers began smoking before the age of 18 (SAMHSA, 2011), and alcohol is typically first consumed before 16 years of age (Behrendt *et al.*, 2009). Important age and sex interactions are also present, as adolescent women are more likely than their male peers to start smoking and less likely to successfully quit (Perkins *et al.*, 1999; Anderson & Burns, 2000). However, rates of e-cigarette use may be higher among adolescent males than females (Kong *et al.*, 2017).

Despite rates of overall drug use declining in recent decades, co-use of nicotine and alcohol by 12th graders increased between 1976-2010 (Daw *et al.*, 2013). Indeed, even occasional smoking in young adults is associated with more frequent drinking, and there is significantly higher alcohol consumption when smoking and drinking occur together (Harrison & McKee, 2011; Silveira *et al.*, 2018). There have been alarming increases in teen e-cigarette use (Johnston *et al.*, 2018), as well as a greater risk of

excessive alcohol consumption, binge drinking, and AUDs among young e-cigarette users (Soneji *et al.*, 2017; Silveira *et al.*, 2018). Coupled with the growing body of evidence demonstrating the unique effects of concurrent versus individual exposure to nicotine and alcohol (Chapter 2; Cross *et al.*, 2017), understanding the mechanisms underlying adolescents' unique vulnerability to nicotine and alcohol co-use is imperative to public health efforts.

The kappa opioid receptor (KOR) and its endogenous ligand, dynorphin, are widely distributed in the human and rodent brain. As a result, they are poised to play a substantial role in regulating motivation and drug reward, executive function, and stress responsiveness (Chavkin *et al.*, 1982; Mansour *et al.*, 1995). Although KORs appear early in ontogeny (Kornblum *et al.*, 1987), recent evidence suggests that functional maturation continues throughout adolescence, with adolescents generally being less sensitive to the effects of KOR activation than adults (Tejeda *et al.*, 2012a; Anderson *et al.*, 2014). Female humans and rodents are also less sensitive to the analgesic and aversive effects of KOR agonists than males (Chartoff & Mavrikaki, 2015), which suggests that the dynorphin/KOR system may have distinct roles depending on sex and age. Indeed, our lab recently demonstrated that KORs age- and sex-dependently modulate self-administration of concurrent nicotine and alcohol (Nic+EtOH). That is, intravenous Nic+EtOH is more reinforcing in adolescent males compared to adult rats of both sexes, but KOR blockade with the selective antagonist, norbinaltorphimine (norBNI), enhances Nic+EtOH self-administration only in adult males, while having no effect in adult females or adolescents of either sex (Lárraga *et al.*, 2017).

These data suggest that Nic+EtOH may induce dynorphin release and subsequent KOR activation that inhibits drug reinforcement in adult males. To further examine the role of KORs in regulating behavioral responses to nicotine and alcohol across development and between sexes, in this chapter I examined how KOR blockade affects acquisition of intravenous nicotine and ethanol self-administration and drug-induced anxiety-like behavior in adolescent and adult rats of both sexes. To explore whether observed behavioral effects are explained by differences in KOR expression and/or function, I measured regional KOR mRNA expression and functional receptor activity in drug naïve animals at time points matching those of our behavioral experiments. My findings highlight the complex role KORs play in regulating behavioral responses to nicotine and alcohol across development in males and females.

Materials and Methods

Animals

Male and female Sprague Dawley rats were obtained from Charles River (Hollister, CA) at postnatal day (P) 17-18 with dams or at P75-76 for behavioral experiments, or P24 and P82 for biochemical experiments. Adults and juveniles weaned at P21 were pair housed by sex in an AALAC-accredited vivarium on a 12-hour light-dark cycle with food and water available *ad libitum*. No more than one animal per litter per experimental group was used to avoid potential litter effects. All procedures were in compliance with NIH guidelines and were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine. Animals were handled for 2 min daily for three days prior to surgery and thereafter, or for the three days prior to tissue collection. All

behavioral testing occurred during the light cycle. Estrous cycle was not measured, as ovarian hormones have not been implicated in KOR-mediated aversion or anxiety- and depressive-like behaviors (Abraham *et al.*, 2018).

Drugs

Nicotine hydrogen tartrate was purchased from Sigma Aldrich (St. Louis, MO), 100% ethanol was purchased from Gold Shield Distributors (Hayward, CA), norBNI from Tocris Biosciences (Minneapolis, MN), and Propofol from Abbot Laboratories (Chicago, IL). All drugs were dissolved in saline and filtered through sterile filters (Millipore Millex Sterile Filters, 0.22 μm pore, 3.3 mm diameter). Nicotine doses (pH=7.4) were calculated as free base, and EtOH was prepared at concentrations no greater than 20% EtOH (v/v).

Catheterization

Adolescent and adult rats underwent catheter implantation surgery at P28 and P86, respectively. Animals were anesthetized with Equithesin (0.035 mg/kg, i.p.), and surgically implanted with a catheter into their right jugular vein. The following day, animals were pretreated with the irreversible kappa opioid receptor antagonist, norBNI (0 or 10 mg/kg, i.p.). Cannulae were flushed daily with heparinized saline to maintain catheter patency. Propofol (5 mg/kg, i.v.) was injected before testing and at the conclusion of self-administration; data were discarded from animals that did not display rapid (5-10 sec) anesthesia.

Behavioral testing

Intravenous self-administration

Starting at P32 or P90 rats (n=7-12/group) initiated intravenous self-administration of nicotine (7.5 μg/kg/infusion) or ethanol (1 mg/kg/infusion) for three days. Animals that self-administered nicotine (7.5 μg/kg/infusion) + ethanol (1 mg/kg/infusion) were processed in parallel, results of which have been published previously (Lárraga *et al.*, 2017). Animals were placed in an operant chamber equipped with a house light and two nose-poke holes with cue lights directly above. Reinforced and non-reinforced responses were recorded, although responses in the non-reinforced hole had no consequence. Each session lasted 2 hr and animals responded at a fixed ratio (FR) 1 reinforcement schedule with a 3 sec timeout between infusions. Drug reinforcement was indicated by significant differences between responding at reinforced and non-reinforced holes.

Drug-induced locomotor activity

On P32 or P90, rats (n=6-11/group) were allowed to habituate to the experimental room for 30 min in their home cages. Animals received two intravenous injections of saline (1 ml/kg), nicotine (2 x 15 μg/kg), ethanol (2 x 2 mg/kg) or Nic+EtOH (2 x 15 μg/kg nicotine plus 2 mg/kg EtOH) spaced one minute apart. Immediately after treatment, animals were placed in a novel open-field activity chamber (43.38 x 43.38 x 30.28 cm) connected to a common interface and computer (Med Associates Inc., St. Albans, VT) for 30 min. Ambulatory counts and time spent in the center of the open-field were recorded automatically.

Tissue collection

Brains from experiment naïve rats were collected on P32 or P90 and snap-frozen in isopentane at -20°C before storage at -80°C until further processing for *in situ* hybridization or [^{35}S]GTPγS binding autoradiography.

[^{35}S]GTPγS autoradiography

Twenty µm coronal sections were cut on a cryostat at -20°C and thaw mounted onto Superfrost Plus microscope slides. Sections were stored at -20°C overnight before processing the next day. Sections were thawed at room temperature for 30 min before equilibrating in assay buffer (50 mM Tris-HCl, 3 mM MgCl2, 0.2 mM EGTA, 100 mM NaCl; pH=7.4; RT) for 10 min. Sections were pre-incubated in assay buffer containing 5'-guanylate diphosphate (2 mM GDP) and dipropylcyclopentylxanthine (1 µM DPCPX) for 15 min at RT. Agonist-stimulated KOR activity was then determined by incubating tissue sections in [^{35}S]GTPγS (40 pM) with the selective KOR agonist U69,593 (10 µM) for 75 min at RT. The incubation was stopped by two 5 min washes in ice-cold wash buffer (50 mM Tris HCl; pH=7.4), followed by a brief rinse in ice-cold ddH_2O. Sections were air dried at RT for at least 1 hr and apposed to Kodak Biomax MR film together with [^{14}C] standards for 48 hrs before being developed with Kodak GBX Developer and RapidFix solutions.

In situ hybridization

Twenty μm coronal sections were cut on a cryostat at -20°C and thaw mounted onto Superfrost Plus microscope slides. Sections were fixed with 4% paraformaldehyde for 1 hr at room temperature followed by 3 x 5 min washes in 0.1 M phosphate-buffered saline. All slides were dried with desiccant and stored at -20°C until processing. [^{35}S]-labeled UTP (Perkin Elmer, Boston, MA) was used to synthesize cRNA riboprobes for the rat KOR in the sense and antisense orientation from a pBS-KS plasmid containing a 1374 bp fragment of rKOR cDNA (kindly provided by Dr. Stanley J. Watson, University of Michigan) between T3 and T7 promoter sites.

Fixed tissue sections were pretreated with Proteinase K (1 μg/ml) for 10 min at 22°C, acetylated, dehydrated through ascending concentrations of ethanol and then air dried. Sections were incubated for 16-18 hr at 60°C with hybridization solution (50% formamide, 10% dextran sulfate, 0.02% Ficoll, 0.02% polyvinyl pyrolidone, 0.02% bovine serum albumin, 500 μg/ml tRNA, 10 mM DTT, 0.3 M NAcCl, 10 mM Tris pH 8.0, 1 mM EDTA pH 8.0) containing the [^{35}S]-labeled riboprobe (10^7 cpm/μl). Following hybridization, sections were incubated with RNase A (20 μg/ml) for 30 min at 37°C, and washed twice at 22°C for 5 min each with 2X SSC buffer/10 mM DTT and 1X SSC buffer/10 mM DTT buffer, followed by a 30 min wash in 1X SSC at 60ºC and a brief rinse in 1X SSC at RT. Tissue sections were dehydrated in ethanol and apposed to Kodak Biomax MR film together with [^{14}C] standards for 72 hrs before being developed with Kodak GBX Developer and RapidFix solutions.

Anatomical analysis

Digitized autoradiograms were quantified with a MicroComputer Imaging Device (MCID Imaging Research, St. Catherine, Ontario, Canada). A calibration curve of optical density against radioligand concentration (dpm/mg tissue) was constructed using [^{14}C] brain paste standards (Broide *et al.*, 1995). Optical densities were measured from 2-3 sections containing each region of interest and values of radioactivity were calculated by interpolation from the calibration curve and averaged to give the value for a single region. For agonist-stimulated [^{35}S]GTPγS binding, nonspecific binding of [^{35}S]GTPγS was subtracted from both basal and specific binding. Specific binding was expressed as percent above basal binding. For *in situ* hybridization, specific hybridization was calculated by subtracting values of radioactivity in sections hybridized with sense probe from those hybridized with antisense probe.

Brain areas in autoradiograms were identified with well-defined anatomical landmarks in reference to adjacent brain sections processed for Nissl-staining. Regions of interest for analysis were: prefrontal cortex [lateral orbital cortex (LO), cingulate cortex (Cg1), prelimbic cortex (PrL), and infralimbic cortex (IL)], claustrum (CL), striatum [dorsomedial caudate putamen (dmCPu), dorsolateral caudate putamen (dlCPu), ventromedial caudate putamen (vmCPu), ventrolateral caudate putamen (vlCPu), nucleus accumbens shell (NAc-shell), and nucleus accumbens core (NAc-core)], bed nucleus of the stria terminalus [anterior (aBNST) and posterior (pBNST)], paraventricular nucleus of the hypothalamus (PVH), paraventricular nucleus of the thalamus (PVT), amygdala [medial nucleus (MeA), central nucleus (CeA), and basolateral (BLA)], hippocampus [CA1, CA2, CA3, and dentate gyrus (DG)], substantia

nigra [pars compacta (SNc) and reticulata (SNr)], ventral tegmental area [anterior (aVTA) and posterior (pVTA)], and raphe nucleus [dorsal (DR) and median (MR)].

Statistical analyses

All data are expressed as mean + SEM. For self-administration, mean reinforced and non-reinforced responses averaged over the last two days were analyzed by five-way ANOVA for Age x Sex x Drug x Pretreatment x Responses (reinforced/non-reinforced), with repeated measures on Responses. Locomotor and center time data were analyzed separately by four-way ANOVA for Age x Sex x Pretreatment x Drug. Regional mRNA expression and [^{35}S]GTPγS binding data were analyzed separately by region using a two-way ANOVA for Age x Sex. All significant main or interaction effects were further analyzed by one-way ANOVA with Bonferroni-adjusted *post hoc* comparisons (SPSS 25.0, Chicago, IL).

Results

KORs age- and sex-dependently regulate nicotine or alcohol reinforcement

There were significant age and sex differences in the acquisition of self-administration of nicotine or ethanol (Figure 3.1). Since overall ANOVA indicated significant Age*Sex*Drug*Pretreatment*Response interaction ($F_{1,130}$=13.330, p<0.001), data were split by drug and analyzed separately for each sex.

In males, ethanol responding was significantly influenced by age and pretreatment, as indicated by a main effect of Age ($F_{1,39}$=22.495, p<0.001) and significant Pretreatment*Response ($F_{1,39}$=5.960, p=0.019) and

Pretreatment*Age*Response ($F_{1,39}$=4.847, p=0.034) interactions. Ethanol was reinforcing in both vehicle- and norBNI-pretreated adult males, as indicated by a significant difference between reinforced and non-reinforced responses (p<0.01). Whereas ethanol alone was not reinforcing in control adolescent males, KOR blockade increased reinforced responding for ethanol in this age group (p=0.030; Figure 3.1A) and induced a significant difference between reinforced and non-reinforced responses (p=0.003). In females self-administering ethanol, there were Age*Pretreatment ($F_{1,26}$=4.770, p=0.038) and Pretreatment*Age*Response ($F_{1,26}$=5.961, p=0.022) interactions. Ethanol was reinforcing in adolescent female controls (p<0.01), and norBNI did not significantly reduce reinforced responding in this group. In contrast, there was a significant main effect of Pretreatment ($F_{1,12}$=4.967, p=0.046) and a significant Pretreatment*Response interaction ($F_{1,12}$=, p=0.036) for ethanol self-administration in adult females. Whereas ethanol was not self-administered by adult female controls, KOR blockade increased reinforced responding for ethanol (p=0.045), and only norBNI-pretreated adult females found ethanol reinforcing (p=0.040; Figure 3.1B).

Both adolescent and adult males found nicotine reinforcing (p<0.001), regardless of norBNI pretreatment (Figure 3.1C). Nicotine responding was higher in control adolescents than adults (p=0.012), as our lab have shown previously (Gellner *et al.*, 2016). Although nicotine reinforced responses were not altered by norBNI pretreatment in adolescent females, in adult females there was a main effect of Pretreatment ($F_{1,11}$=21.102, p=0.001) and a Pretreatment*Response interaction ($F_{1,11}$=9.962, p=0.010). Nicotine was self-administered by adult female controls (p=0.019), an effect

that was blocked by norBNI-pretreatment. KOR blockade significantly decreased reinforced responding for nicotine in adult females (p=0.007; Figure 3.1D).

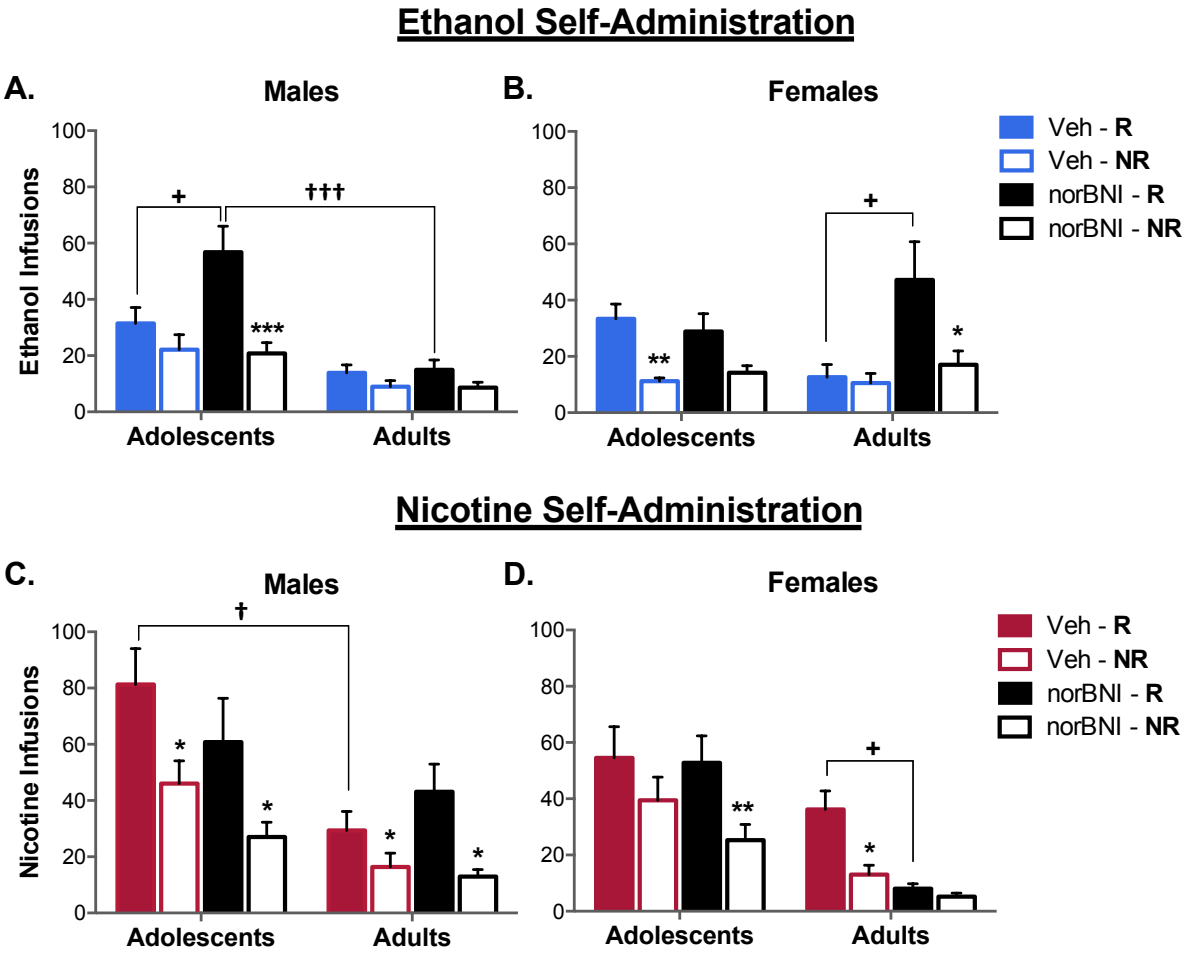

Figure 3.1. Acquisition of ethanol, but not nicotine, self-administration is enhanced by KOR blockade in adolescent males and adult females. (A) norBNI increased responding for ethanol in adolescent, but not adult, males compared to vehicle treatment. (B) Treatment with norBNI increased responding for EtOH in adult, but not adolescent, females compared to vehicle treatment. (C) Males self-administered nicotine regardless of pretreatment, but adolescent males self-administered more nicotine than adult males. (D) Responding for nicotine is decreased by norBNI in adult females. Data are expressed as mean + SEM. R = reinforced responses; NR = nonreinforced responses; EtOH = ethanol; Veh = vehicle. *, p<0.05, ***, p<0.001 vs. R; +, p<0.05 vs. Veh; †, p<0.05, †††, p<0.001 vs. adults. n = 6-12/group.

Nic+EtOH is anxiolytic in adult females via a KOR-dependent mechanism

Drug-induced anxiety-like behavior, as measured by time spent in the center of a novel open-field, was age- and sex-dependently regulated by KORs (Figure 3.2). I

observed significant main effects of Sex ($F_{1,251}$=47.707, p<0.001) and Pretreatment ($F_{1,251}$=12.475, p<0.001), and a significant Pretreatment*Drug interaction ($F_{3,251}$=3.129, p=0.026). Data were split by sex for further analysis. There were no significant effects of drug or KOR blockade on center time in males (Figure 3.2A,B).

In females, there was a main effect of Pretreatment ($F_{1,127}$=13.643, p<0.001) and significant Age*Drug ($F_{3,127}$=2.983, p=0.034) and Pretreatment*Drug ($F_{3,127}$=2.989, p=0.034) interactions. Saline-treated adolescent females spent significantly more time in the center zone than their adult female counterparts (p=0.004). Ethanol decreased center time (p=0.033) compared to saline in both vehicle- and norBNI-pretreated adolescent females (Figure 3.2C). In contrast, Nic+EtOH increased center time in adult female controls (p=0.006), an anxiolytic effect that was blocked by norBNI pretreatment (p<0.001; Figure 3.2D).

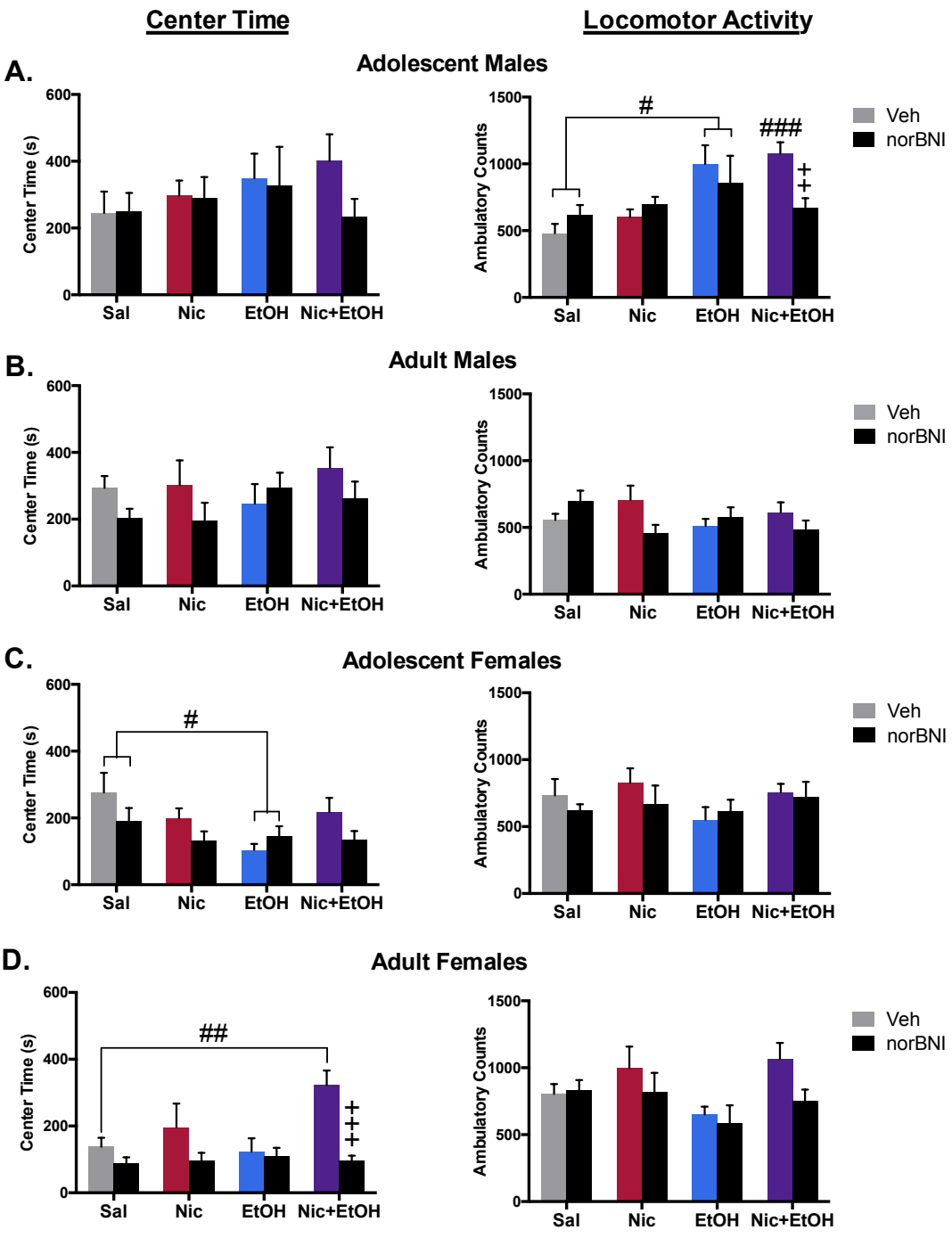

Figure 3.2. Age and sex differences in KOR modulation of locomotor and anxiety-like behavior. (A) Center time is not significantly affected by drug or KOR blockade in adolescent males. EtOH and Nic+EtOH increase locomotor activity in adolescent males; norBNI blocks Nic+EtOH's effect, without affecting ethanol's effect. (B) No significant effects were observed in adult males. (C) EtOH decreases center time in adolescent females, regardless of pretreatment, without affecting locomotor activity. (D) Nic+EtOH increased the time adult females spend in the center of an open-field, indicating an anxiolytic effect. This was blocked by norBNI, and locomotor was not significantly affected by any drug. Data are expressed as mean + SEM. Sal = saline; Nic = nicotine; EtOH = ethanol; Nic+EtOH = nicotine and ethanol; Veh = vehicle. #, $p<0.05$, ##, $p<0.01$, ###, $p<0.001$ vs. Sal; ++, $p<0.01$, +++, $p<0.001$ vs. Veh. N = 6-10/group.

Nic+EtOH increases locomotion in adolescent males via a KOR-dependent mechanism

I observed significant age and sex differences in the locomotor stimulating effects of nicotine and/or ethanol (Figure 3.2). There were significant main effects of Sex ($F_{1,251}$=6.575, p=0.011) and Pretreatment ($F_{1,251}$=5.319, p=0.022), as well as significant interactions between Age*Sex ($F_{1,251}$=19.337, p<0.001), Age*Drug ($F_{3,251}$=3.405, p=0.018), Sex*Drug ($F_{3,251}$=4.945, p=0.002), and Pretreatment*Drug ($F_{3,251}$=3.096, p=0.028). Data were split by sex for further analysis.

In males, I observed significant main effects of Age ($F_{1,124}$=17.326, p<0.001) and Drug ($F_{3,124}$=2.960, p=0.035), as well as significant Age*Drug ($F_{3,124}$=6.633, p<0.001), Pretreatment*Drug ($F_{3,124}$=4.075, p=0.008), and Age*Drug*Pretreatment ($F_{3,124}$=2.819, p=0.042) interactions. In adolescent males, both ethanol (p=0.024) and Nic+EtOH (p<0.001) increased locomotor activity. The locomotor stimulatory effect of Nic+EtOH, but not of ethanol, was blocked by norBNI (p=0.008; Figure 3.2A). In adult males, there was a Pretreatment*Drug ($F_{3,69}$=3.047, p=0.034) interaction, but no significant group differences remained after post-hoc correction (Figure 3.2B).

In females, there were significant main effects of Age ($F_{1,127}$=5.517, p=0.020) and Drug ($F_{3,127}$=3.780, p=0.012), but no significant group differences remained after post-hoc analysis (Figure 3.2C,D).

Functional KOR activity differs in an age-, sex-, and brain region-dependent manner

There were significant age, sex, and regional differences in functional KOR activity in the brains of drug naïve rats (Figure 3.3). In the PVH, there were main effects of Age ($F_{1,38}$=4.092, p=0.050) and Sex ($F_{1,38}$=7.502, p=0.009). Post hoc analyses revealed that adult females had greater U69,593-stimulated [^{35}S]GTPγS binding than adolescent females (p=0.034), adult males (p=0.002), and adolescent males (p=0.001; Figure 3.3A). Similar results were observed for all subregions of the amygdala (Figure 3.3B-D). In the MeA, there were main effects of Age ($F_{1,43}$=9.093, p=0.004), Sex ($F_{1,43}$=13.637, p=0.001), and an Age*Sex interaction ($F_{1,43}$=4.428, p=0.041), with adult females having higher KOR activity than adolescent females (p=0.019), adult males (p=0.002), and adolescent males (p<0.001). In the CeA, there were main effects of Age ($F_{1,44}$=7.724, p=0.008), Sex ($F_{1,44}$=5.534, p=0.023), and an Age*Sex interaction ($F_{1,44}$=7.159, p=0.010). Adult females had higher [^{35}S]GTPγS binding in the CeA than adolescent females (p=0.006), adult males (p=0.007), and adolescent males (p=0.001). In the BLA, there was a main effect of Age ($F_{1,43}$=12.804, p=0.001) and an Age*Sex interaction ($F_{1,43}$=10.238, p=0.003). Post hoc analyses showed that adult females had higher KOR activity than adolescent females (p=0.003), adult males (p=0.004), and adolescent males (p=0.002).

In contrast, there were significant Age*Sex interactions in the VTA ($F_{1,44}$=4.457, p=0.040) and MR ($F_{1,47}$=4.316, p=0.044). KOR activity was significantly higher in adolescent than adult males both in the VTA (p=0.048) and MR (p=0.011; Figure 3.3E,F).

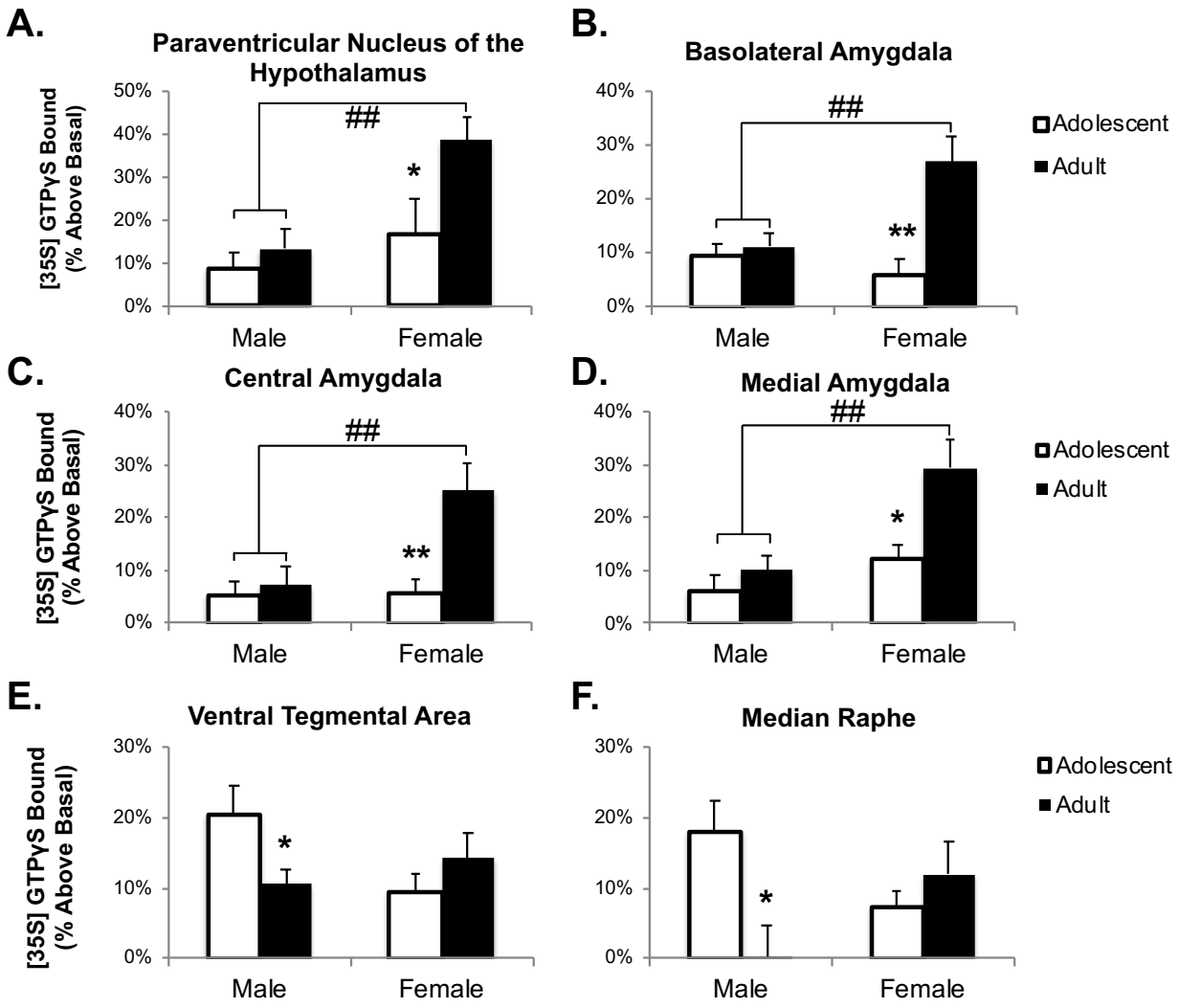

Figure 3.3. Functional activity of KORs in drug naïve rats varies by age, sex, and brain region. Adult females have higher U69,593-stimulated [^{35}S]GTPγS binding than males or adolescent females in the paraventricular nucleus of the hypothalamus (A), as well as in the basolateral (B), medial (C), and central (D) nuclei of the amygdala. Adolescent males have higher functional KOR activity in the ventral tegmental area (E) and median raphe (F) than adult males. Data are expressed as mean + SEM. *, $p<0.05$, **, $p<0.01$ vs. adults; ##, $p<0.01$ vs. females. n = 6-18/group.

Regional KOR mRNA expression

Regional KOR mRNA expression was significantly influenced by age and sex in drug naïve rats (Figure 3.4). There were main effects of Age in the medial ($F_{1,22}$=4.648, p=0.042) and basolateral ($F_{1,23}$=12.402, p=0.002) nuclei of the amygdala, with adolescents having higher KOR mRNA expression than adults, regardless of sex

(Figure 3.4A,B). There were main effects of Sex in the MR ($F_{1,22}$=6.117, p=0.022) and pBNST ($F_{1,19}$=6.924, p=0.016), where females had higher mRNA expression than males (Figure 3.4C,D). In contrast, there were main effects of Age ($F_{1,20}$=10.800, p=0.004) and Sex ($F_{1,20}$=4.593, p=0.045) in the aBNST, with adult females having significantly higher KOR mRNA expression than adolescent females (p=0.042; Figure 3.4E).

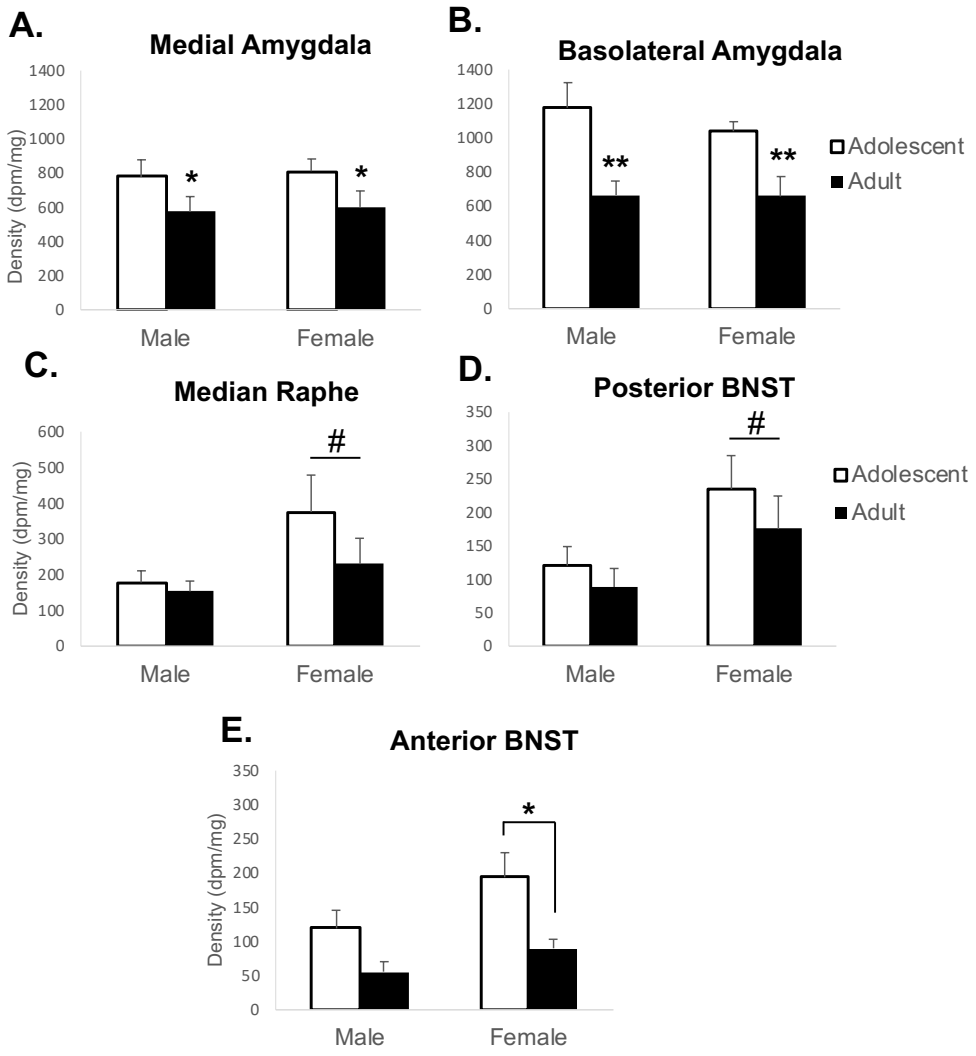

Figure 3.4. KOR mRNA expression in drug naïve rats varies by age, sex, and brain region. Adults of both sexes have lower mRNA expression than adolescents in the medial (A) and basolateral (B) nuclei of the amygdala. Females, regardless of age, had higher KOR mRNA expression in the posterior BNST (C) and median raphe (D). In contrast, adult females have higher KOR mRNA expression than males or adolescent females in the anterior BNST (E). Data are expressed as mean + SEM. * p<0.05, ** p<0.01 vs. adolescents; # p<0.05 vs. males. n = 5-8/group.

Discussion

A wealth of literature has linked the dynorphin/KOR system with modulation of behavioral and neurochemical responses to chronic drug exposure (Wee & Koob, 2010; Crowley & Kash, 2015; Anderson & Becker, 2017). However, investigation into KOR-mediated regulation of the acute effects of nicotine and alcohol has been lacking. This is particularly noticeable for females and during sensitive developmental periods such as adolescence, despite high rates of nicotine and alcohol consumption and dependence among these groups. Using an acquisition of self-administration paradigm, our lab has previously shown both sex- and age-dependent modulation by KORs of the reinforcing effects of a nicotine + alcohol combination (Lárraga *et al.*, 2017; Table 3.1). We now show that KORs also modulate the initial reinforcing effects of nicotine and alcohol alone, but with a different sex- and age-dependent profile. I also show that anxiety-like and locomotor behaviors following acute drug exposure are age- and sex-dependently altered by KOR manipulation, and demonstrate significant differences in KOR functional activity and mRNA expression in brain regions critically involved in motivated behavior and stress response. Together, these data highlight the complexity of KOR signaling across sex and development.

Self-administration

Here, we provide the first evidence for KOR signaling playing distinct modulatory roles in the initial reinforcing effects of nicotine or alcohol based on age, sex, and self-administered drug. In control animals, low dose ethanol (1 mg/kg/inf.) had a mild reinforcing effect in adult males and adolescent females (Table 3.1). Pretreatment with

the KOR antagonist, norBNI, had no effect on ethanol self-administration in adult males, a finding in agreement with other studies using norBNI in non-dependent adult males (Walker & Koob, 2008; Walker *et al.*, 2011). Similarly, norBNI was without effect in adolescent females. In contrast, acquisition of ethanol self-administration was strongly facilitated in adolescent males and adult females by pretreatment with norBNI. This finding suggests that KOR activation tonically inhibits ethanol reinforcement in these groups. This contrasts with prior studies that have shown females and adolescents to be generally less sensitive to the effects of KOR activation (Anderson *et al.*, 2013; Anderson *et al.*, 2014), and that norBNI decreases oral ethanol intake in adult female rats (Morales *et al.*, 2014). However, no prior studies have examined initial reinforcing effects of these drugs in a self-administration acquisition paradigm, as was done in the current study.

Nicotine self-administration was also age- and sex-dependently influenced by KOR manipulation. Our data do not support a contribution of KORs in regulating acquisition of nicotine self-administration in males or adolescent females. In males, nicotine was found to be reinforcing in both age groups, although reinforced responses at this low drug dose were significantly higher in adolescents than adults, as we have previously shown (Gellner *et al.*, 2016). KOR blockade by norBNI did not significantly impact reinforced responding for nicotine in males, consistent with prior findings (Liu & Jernigan, 2011). Although reinforced responding for nicotine in adolescent females was unaffected by norBNI pretreatment, it was significantly decreased in adults (Table 3.1). This unexpected finding would suggest that KOR activation *contributes* to the initial reinforcing properties of nicotine in adult females.

Table 3.1. Summary of norBNI effects on self-administration of nicotine, ethanol, or Nic+EtOH.

Self-administered drug		Males		Females	
		Reinforcing?	Change in reinforced responding	Reinforcing?	Change in reinforced responding
Nicotine	Adolescent	Yes	=	No	↑
	Adult	Yes	=	Yes	=
Ethanol	Adolescent	No	↑	Yes	↓
	Adult	Yes	=	No	↑
Nic+EtOH	Adolescent	Yes	=	Yes	=
	Adult	No	↑	No	=

Reinforcing is defined as a significant difference between reinforced and non-reinforced nose-poke hole responding during initial 3-day acquisition of drug self-administration. Arrows and equal signs represent change or insensitivity, respectively, in response norBNI pretreatment on reinforced responding. Nic+EtOH data are summarized from Lárraga *et al*., 2017.

Whereas KORs also play a role in modulating the reinforcing effects of combined Nic+EtOH (Lárraga *et al.*, 2017; Table 3.1), this is distinct from that of either drug alone. NorBNI pretreatment enhances the reinforcing effect of Nic+EtOH in adult males, in contrast to the enhancement of ethanol intake in adolescent males. Furthermore, whereas norBNI has no effect on Nic+EtOH self-administration in females, it has significant and opposite effects on female intake of ethanol or nicotine alone. Although nicotine and ethanol share many neurochemical and molecular targets (Cross *et al.*, 2017; Klenowski & Tapper, 2018), these data further highlight the complex interactions

of nicotine and/or ethanol with KOR signaling in males versus females and suggest that distinct, drug-specific anatomical pathways may be involved in modulating the reinforcing properties of each drug.

My findings also complement epidemiological and clinical literature indicating differential effects of individual versus concurrent nicotine and alcohol use. For example, cessation success is negatively impacted by concurrent use (McKee & Weinberger, 2013; Weinberger *et al.*, 2013; Chiappetta *et al.*, 2014; Weinberger *et al.*, 2015), and the rewarding value of alcohol can be enhanced by nicotine, and vice versa (Glautier *et al.*, 1996; Kouri *et al.*, 2004; Rose *et al.*, 2004; King & Epstein, 2005). Sex differences are also present, as female smokers report higher levels of craving for alcohol in treatment settings than male smokers or nonsmoking females (Hitschfeld *et al.*, 2015). Mechanisms underlying these effects are not well understood, but our findings emphasize the importance of studying the unique properties of concurrent versus individual nicotine and alcohol exposure.

Anxiety and locomotion

KOR modulation of other behaviors also showed drug-, sex-, and age-related disparities. Using time spent in the center of an open-field to examine anxiety-like behavior, I have shown that ethanol decreased anxiety in adolescent females, but this was not mediated by KORs since norBNI pretreatment had no effect. In contrast, concurrent Nic+EtOH produced a significant anxiolytic effect in adult females which was blocked by norBNI pretreatment, indicating a KOR mechanism. This finding was unexpected, given a substantial literature that KOR activation produces anxiety and

depression, particularly in males (Van't Veer & Carlezon, 2013). However, anxiolytic effects of KOR agonists have been seen (Privette & Terrian, 1995; Braida *et al.*, 2009; Alexeeva *et al.*, 2012).

Adult males did not show any change in anxiety or locomotor response as a result of drug treatment. Whereas there were no significant drug-induced effects on anxiety-related behavior in adolescent males, in contrast to what was observed in Chapter 2, there were significant effects on general locomotion. Both ethanol and Nic+EtOH increased ambulatory counts; however, norBNI only reversed the effects of the latter. Thus, as with other behaviors examined, there is a complex link between drug effects and KOR activity.

Neuroanatomy

In order to examine possible neuroanatomical correlates of age and sex differences in KOR function, I measured KOR mRNA expression and G-protein coupling in brain areas critical for mood and motivated behaviors. U69,593-stimulated [^{35}S]GTPγS binding was found to be higher in the VTA and MR of adolescent males than adults. Adult females also have greater functional KOR activity in the PVH and amygdala than males or adolescent females. The amygdala plays a key role in mood, as well as in interactions between drug use and stress (Janak & Tye, 2015). KOR activation in the amygdala has been implicated in aversion (Zan *et al.*, 2016), and in anxiety-like behaviors during withdrawal from chronic drug exposure. Increased KOR activity in these areas may be due to age and sex differences in G-protein coupling efficacy, but may also result from differences in KOR density. However, I have found

that early adolescent rats (P32) have higher KOR mRNA expression in the MeA and BLA than adults, regardless of sex. In contrast, females have higher KOR mRNA levels in the MR and posterior BNST than males, and adolescent females have enhanced KOR expression in anterior BNST compared to other groups. Thus, the age- and sex-dependent changes in regional [^{35}S]GTPγS binding that I observed are not paralleled by changes in KOR mRNA expression.

Conclusion

The present findings add to a growing literature on complex regulation of drug-induced behavior by KORs. Most studies of addictive drugs examine KOR modulation of their chronic effects. My data suggest that the reinforcing effects of initial drug exposure are also regulated by KORs. Furthermore, whereas most studies show anxiogenic and hypolocomotor effects of KOR activation (Van't Veer & Carlezon, 2013), I have shown opposite effects that are dependent on drug, sex, and age. These data also highlight the unique behavioral effects of concurrent versus individual exposure to nicotine and ethanol, and suggest that KORs have distinct functional roles in modulating behavioral responses to each drug.

Chapter 4

Kappa opioid receptor-mediated regulation of anxiety- and depressive-like behaviors in adolescent and adult rats

Introduction

Stress is a major contributing factor to drug use and dependence. Kappa opioid receptors (KORs) play a critical role in the interaction between stress and substance use, likely through KOR-mediated regulation of motivational and emotional states. Indeed, KORs and their ligand, dynorphin, are found in many brain regions critical for anxiety and stress, including the amygdala, hippocampus, BNST, and PVH (Kornblum *et al.*, 1987; Simonin *et al.*, 1995). Activation of KORs produces aversive and depressive-like behaviors that are thought to mimic the effects of stress (Pfeiffer *et al.*, 1986; Mague *et al.*, 2003; Carlezon *et al.*, 2006). Importantly, this is observed in both humans and rodents. In contrast, KOR antagonists can be anxiolytic and antidepressant (Mague *et al.*, 2003; Shirayama *et al.*, 2004; Knoll *et al.*, 2007; Knoll *et al.*, 2011; Reed *et al.*, 2012). KOR blockade has also been shown to decrease or block stress-induced reinstatement of cocaine-, nicotine-, and alcohol-seeking (Beardsley *et al.*, 2005; Land *et al.*, 2009; Graziane *et al.*, 2013; Funk *et al.*, 2014; Grella *et al.*, 2014; Polter *et al.*, 2014), effects that are thought to be mediated by prevention of stress-induced interactions between dynorphin and KORs that result in aversive and depressive-like responses and subsequent enhancement of drug reinforcement (Bruchas *et al.*, 2009; Bruchas *et al.*, 2010; Grella *et al.*, 2014).

Although there is extensive literature demonstrating a link between KOR signaling and anxiety- or depressive-like behaviors, the contributions of sex and age to this interaction are rarely considered. This is despite a large body of literature demonstrating higher rates of mood disorders in women compared to men (Kessler, 2003; Marcus *et al.*, 2005; Leach *et al.*, 2008; Faravelli *et al.*, 2013), an effect that does not emerge until after puberty (Marcus *et al.*, 2005). There are also major functional differences in KOR activity between males and females (Chartoff & Mavrikaki, 2015). Indeed, female rodents appear to be less sensitive than males to the analgesic and aversive effects of KOR agonists (Barrett *et al.*, 2002; Stoffel *et al.*, 2005; Wang *et al.*, 2011; Russell *et al.*, 2014). Additionally, anxiety-like behavior appears to differ across development, although findings are equivocal and appear to depend largely on age and measure being tested (Desikan *et al.*, 2014). Adolescent male rats also display reduced sensitivity to the dysphoric effects of KOR agonists in conditioned taste aversion and conditioned place aversion paradigms compared to adult males (Anderson *et al.*, 2014). Paradoxical findings of anxiolytic effects of KOR activation have also been reported in male rats that are likely adolescents, based on reported weight (Privette & Terrian, 1995; Braida *et al.*, 2009; Alexeeva *et al.*, 2012).

Very few studies have examined age and sex together as modulators of KOR function, and these have only been done in the context of drug exposure. Morales *et al*., (2014) showed that the KOR antagonist norBNI increased oral alcohol intake in adult males, while decreasing it in adult females, and having no effect on alcohol consumption in adolescents of either sex. In contrast, we have shown that norBNI increases alcohol self-administration in adolescent males and adult females, without

affecting intake in adolescent females or adult males (Chapter 3). Additionally, KOR blockade selectively increases self-administration of concurrent nicotine and ethanol in adult males (Lárraga *et al.*, 2017). However, no study to date has directly examined age and sex as potential regulators of KOR sensitivity in measures of anxiety- and depressive-like behavior. The studies in Chapter 4 therefore use the light-dark test (LDT), elevated plus maze (EPM), and forced swim test (FST) to assess the effects of the selective KOR agonists, U50,488H and U69,593, on anxiety- and depressive-like behavior in adolescent and adult rats of both sexes.

Materials and Methods

Animals

Male and female Sprague Dawley rats were obtained from Charles River (Hollister, CA) at P24 or P82 and group housed by sex in an AALAC-accredited vivarium on a 12-hour light-dark cycle. Food and water were available *ad libitum*. Animals were allowed to acclimate to the vivarium for five days prior to handling. Starting at P29 or P87, animals were handled for 2 min daily for three days prior to behavioral testing at P32 or P90. All procedures were in compliance with NIH guidelines and were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine. All behavioral testing occurred during the light cycle.

Drugs

U69,593 ((+)-(5α,7α,8β)-N-Methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide) was purchased from Sigma Aldrich (St. Louis, MO) and U50,488H

(*trans*-(-)-3,4-Dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide hydrochloride) from Tocris Biosciences (Minneapolis, MN). U69,593 was dissolved in 0.1 M HCl before dilution with sterile saline. U50,488 was dissolved in sterile saline. Both drugs were administered in a volume of 1 ml/kg.

Behavioral testing

Starting at P32 or P90, male and female rats underwent four consecutive days of behavioral testing with either U50,488 or U69,593. The order of testing was identical for all groups and was ordered as follows: light-dark test (LDT), elevated plus maze (EPM), and forced swim test (FST). Each of the three behavioral tests occurred in different rooms and animals were allowed to habituate to the testing room for 30 min in their home cage. Animals were administered U50,488 (0, 3, or 10 mg/kg, i.p.) or U69,593 (0, 0.3, or 3.0 mg/kg, i.p.) 15 min prior to each test. Each rat received the same drug and dose for the entirety of testing, receiving three total injections on days 1, 2, and 4 (no injections were administered during the forced swim test pretest).

Light-dark test (LDT)

Adolescents (P32) and adults (P90) underwent testing of anxiety-like behavior in a light-dark box according to previously published methods (Torres *et al.*, 2015). The light-dark box consists of a novel open-field activity chamber (43.38 x 43.38 x 30.28 cm) with a lidded dark box insert (44.4 x 22.9 x 30.5 cm) containing an open door to allow for movement between the enclosed dark side and the light side. The activity chamber was connected to a common interface and computer (Med Associates Inc., St. Albans, VT).

Following a 30 min habituation and injection with U50,488 or U69,593, animals were placed in the dark side of the apparatus and the lid was closed. Time spent in the light and dark sides of the chamber, as well as distance travelled, were recorded automatically for five minutes. The activity chamber and insert were cleaned after each test.

Elevated plus maze (EPM)

Adolescents (P33) and adults (P91) were subjected to a test in the elevated plus maze according to previously published methods (Daniels *et al.*, 2004; Villégier *et al.*, 2010). The EPM is a plus-shaped acrylic maze with two opposite open arms (50 cm in length and 10 cm in width) and two opposite closed arms (50 cm in length, 10 cm in width, and 31 cm in height) extending out from an open central junction (10 x 10 cm). The entire apparatus is 50 cm above the floor. Following a 30 min habituation and injection with U50,488 or U69,593, animals were placed in the center of the maze facing a closed arm and allowed to explore the apparatus for five minutes. The time spent in each arm or the junction was recorded automatically (Med-PC IV, Med Associates Inc., St. Albans, VT). The maze was cleaned with water after each test.

Forced swim test (FST)

Adolescents (P34) and adults (P92) were subjected to a two-day forced swim test, as described by Porsolt *et al*., (1977). On pretest day, animals were habituated to the testing room for 30 min in their home cage before being placed in a large, clear plastic cylinder (44.45 cm in height and 29.21 cm internal diameter; Med Associates Inc., St.

Albans, VT) filled with room temperature (~25°C) water to a depth of 35 cm. Animals were allowed to swim for 15 min. No behavior was recorded, but all animals were monitored for drowning. At the conclusion of the 15 min, animals were removed, dried thoroughly with paper towels, and placed into a warming cage for 30 min before being returned to their home cage. Twenty-four hours later, the test was repeated with modification as follows: animals received injections of U50,488 or U69,593 15 min prior to testing, and the video camera-recorded session lasted 5 min. Animals were removed, dried, and placed in the warming cage as on pretest day. A blind observer scored behavioral videos for total time immobile, swimming, climbing, or diving.

Data analysis

Data are expressed as mean + SEM. For LDT, distance traveled and time spent in the dark side were analyzed by four-way ANOVA for Agonist x Age x Sex x Dose. For EPM, time spent in the closed arms was analyzed by four-way ANOVA for Agonist x Age x Sex x Dose. For the FST, each behavior was analyzed separately by four-way ANOVA for Agonist x Age x Sex x Dose. All significant main or interaction effects were further analyzed by one-way ANOVA with Dunnett's *post hoc* test (SPSS 25.0, Chicago, IL).

Results

Light-dark test

Behavior in the LDT was not significantly different based on agonist, and data for U69,593 and U50,488-treated animals were therefore combined. There were main effects of Age ($F_{1,135}$=12.593, p=0.001), Sex ($F_{1,135}$=10.530, p=0.001), and Dose

($F_{2,135}$=7.240, p=0.001), as well as a significant Age*Sex interaction ($F_{1,135}$=5.211, p=0.024). Data were split by sex and age for further analysis. In adult females, there was a main effect of Dose ($F_{2,29}$=4.541, p=0.019), with the high dose of agonist increasing time spent in the dark side of the LDT chamber compared to vehicle (p=0.023; Figure 4.1), suggesting an increase in anxiety-like behavior. There were no significant effects of KOR activation in males or adolescent females.

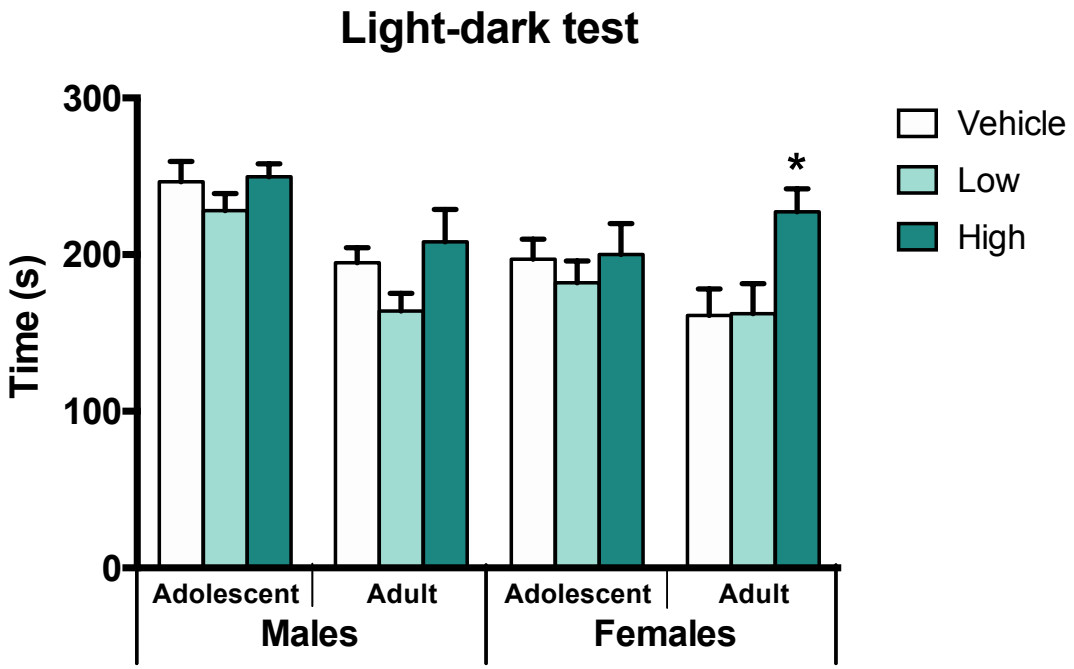

Figure 4.1. Anxiety-like behavior in the LDT is increased by high dose KOR activation in adult females. Time spent in the dark side of the LDT chamber was not significantly different between animals treated with U50,488 or U69,593. However, the high dose of KOR agonist increased time spent in the dark side compared to vehicle in adult females, but not males or adolescent females, suggesting an increase in anxiety-like behavior. Data represent mean + SEM. *, p<0.05 vs. vehicle. n = 11-17/group.

High doses of KOR agonists can have locomotor depressant effects that are attributed to KOR's inhibitory effects on dopamine neurotransmission (Di Chiara & Imperato, 1988; Mague *et al.*, 2003; Brust *et al.*, 2016). To assess whether U50,488 and U69,593 had confounding effects on locomotion at the doses tested, I also measured

distance traveled in the LDT. Data for U69,593 and U50,488 were combined since there were no significant effects of agonist. However, there were main effects of Sex ($F_{1,135}$=27.746, p<0.001), and Dose ($F_{2,135}$=15.144, p<0.001), as well as a significant Age*Sex interaction ($F_{1,135}$=4.331, p=0.039) and a trend for an Age*Sex*Dose interaction ($F_{2,135}$=3.005, p=0.053). Data were split by sex and age for further analysis. In adult males, there was an effect of Agonist ($F_{1,39}$=5.575, p=0.023) and an Agonist*Dose interaction ($F_{2,39}$=16.907, p<0.001), with the high dose decreasing locomotor activity compared to vehicle (p<0.01; Figure 4.2). There were no significant effects in females or adolescent males.

Elevated plus maze

Anxiety-like behavior in the EPM was significantly influenced by age, sex, and dose, but not agonist. Data from U69,593 and U50,488-treated animals were therefore combined. There were main effects of Sex ($F_{1,135}$=9.173, p=0.003) and Dose ($F_{2,135}$=31.100, p<0.001), as well as an Age*Dose interaction ($F_{2,135}$=4.015, p=0.020). Data were split by sex for further analysis. In males, there was a main effect of Dose ($F_{2,77}$=20.347, p<0.001), with the high dose of agonist increasing time spent in the closed arms in both adolescents and adults (p<0.001; Figure 4.3). In females, there were main effects of Agonist ($F_{1,58}$=4.199, p=0.045) and Dose ($F_{2,58}$=12.843, p<0.001), as well as significant interactions of Age*Agonist ($F_{1,58}$=4.149, p=0.046) and Age*Dose ($F_{2,58}$=4.484, p=0.015). In adult, but not adolescent, females the high dose of both agonists increased time spent in the closed arm compared to vehicle, indicating an anxiogenic effect (p=0.002 and p=0.010, respectively; Figure 4.3).

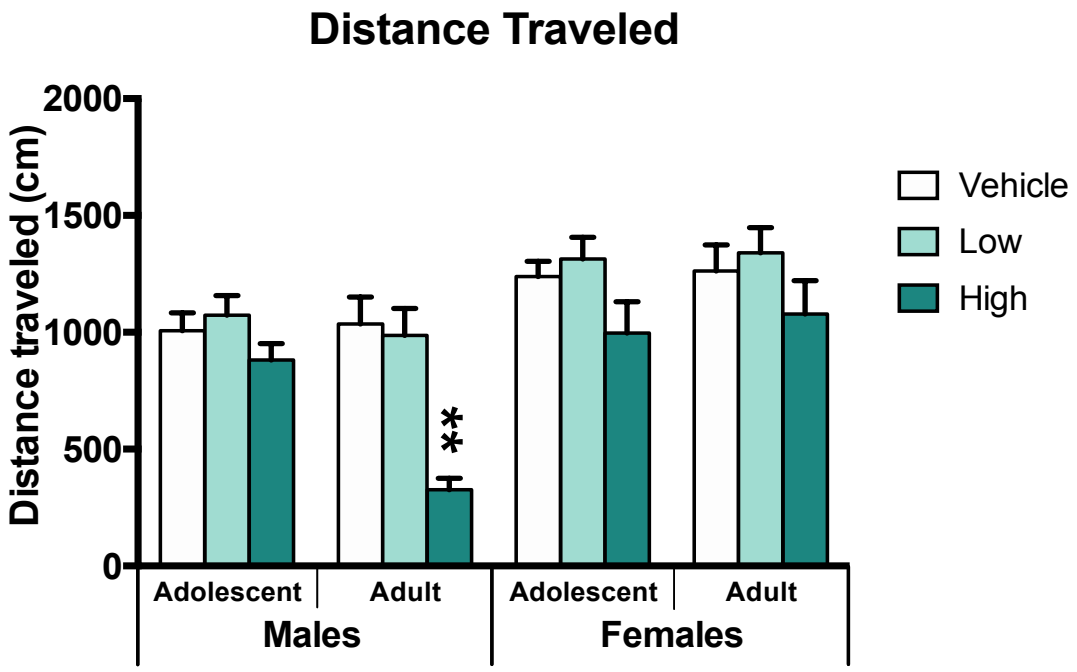

Figure 4.2. KOR agonists have a locomotor depressant effect at high doses in adult males. Distance traveled in the LDT chamber was decreased by the high dose of KOR agonist compared to vehicle in adult males, but not females or adolescent males. Data represent mean + SEM. **, p<0.01 vs. vehicle. n = 11-17/group.

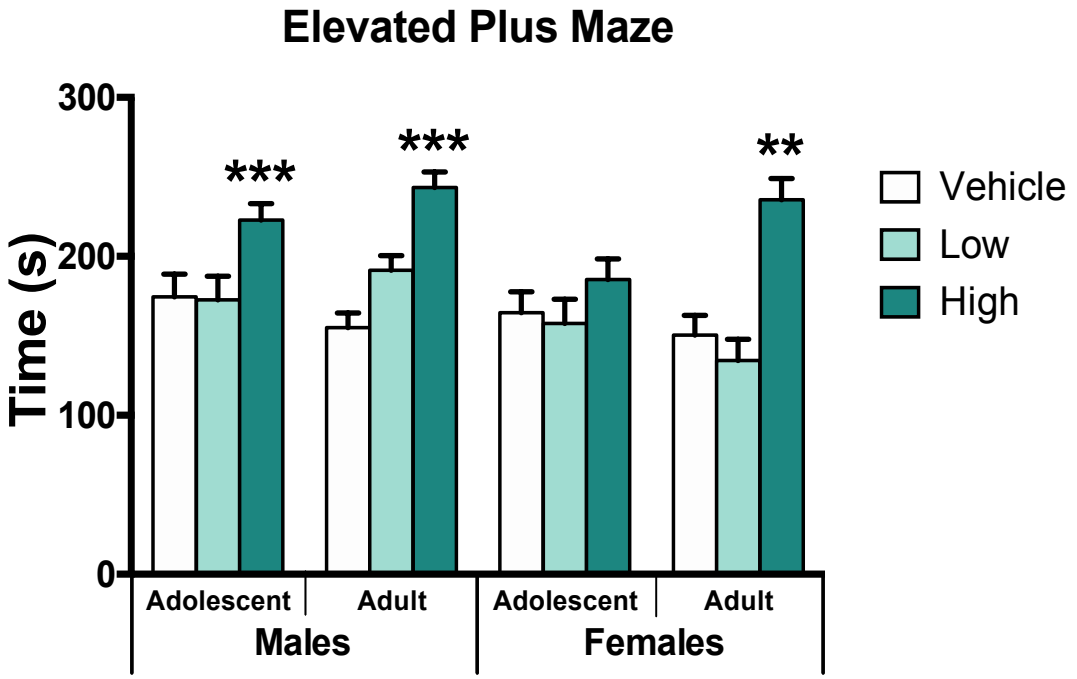

Figure 4.3. KOR activation increases anxiety-like behavior in the EPM of males and adult females. The high dose of both KOR agonists increased time spent in the closed arm of the EPM compared to vehicle, indicating an anxiety-like effect, in adult females and in males at both ages. Data represent mean + SEM. **, p<0.01; ***, p<0.001 vs. vehicle. n = 11-17/group.

Forced swim test

Depressive-like behaviors, as measured by active (i.e., swimming, climbing, and diving) and inactive (i.e., immobility) coping behaviors, were assessed in the FST and analyzed separately (Figure 4.4). For immobility, there were main effects of Age ($F_{1,135}$=17.163, p<0.001) and Dose ($F_{2,135}$=8.262, p<0.001), as well as an Age*Sex*Dose interaction ($F_{2,135}$=3.282, p=0.041). Data were split by age and sex for further analysis. In adolescent males, there was a significant Dose effect ($F_{2,38}$=6.263, p=0.004), with the high dose of KOR agonist increasing immobility compared to vehicle (p=0.028; Figure 4.4A). There was a main effect of Dose ($F_{2,29}$=3.980, p=0.030) in adult females, but there were no significant differences in immobility after post hoc analysis. No significant effects were seen in adult males or adolescent females.

For swimming behavior, there was a main effect of Age ($F_{1,135}$=16.642, p<0.001) and an Age*Sex*Dose interaction ($F_{2,135}$=4.629, p=0.011). In adolescent males, there was a main effect of Dose ($F_{2,38}$=3.557, p=0.038), but no significant effects after post hoc correction. Similarly, there was a Dose ($F_{2,29}$=3.488, p=0.044) effect on swimming in adult females, with a trend (p=0.068) for the low dose of agonist to increase swimming compared to vehicle (Figure 4.4B). Swimming was not significantly influenced by KOR activation in adult males and adolescent females.

There were main effects of Sex ($F_{1,135}$=6.784, p=0.010) and Dose ($F_{2,135}$=9.373, p<0.001) on climbing behavior. After splitting by sex, there was a main effect of Dose ($F_{2,77}$=11.456, p<0.001) in males, with the high dose of the KOR agonists decreasing climbing compared to vehicle (p<0.001; Figure 4.4C). Diving was significantly influenced by Agonist ($F_{1,135}$=4.557, p=0.035) and Dose ($F_{2,135}$=6.899, p=0.001). Compared to

vehicle, the low dose of U69,593 increased diving (p=0.023; Figure 4.4D), regardless of age or sex.

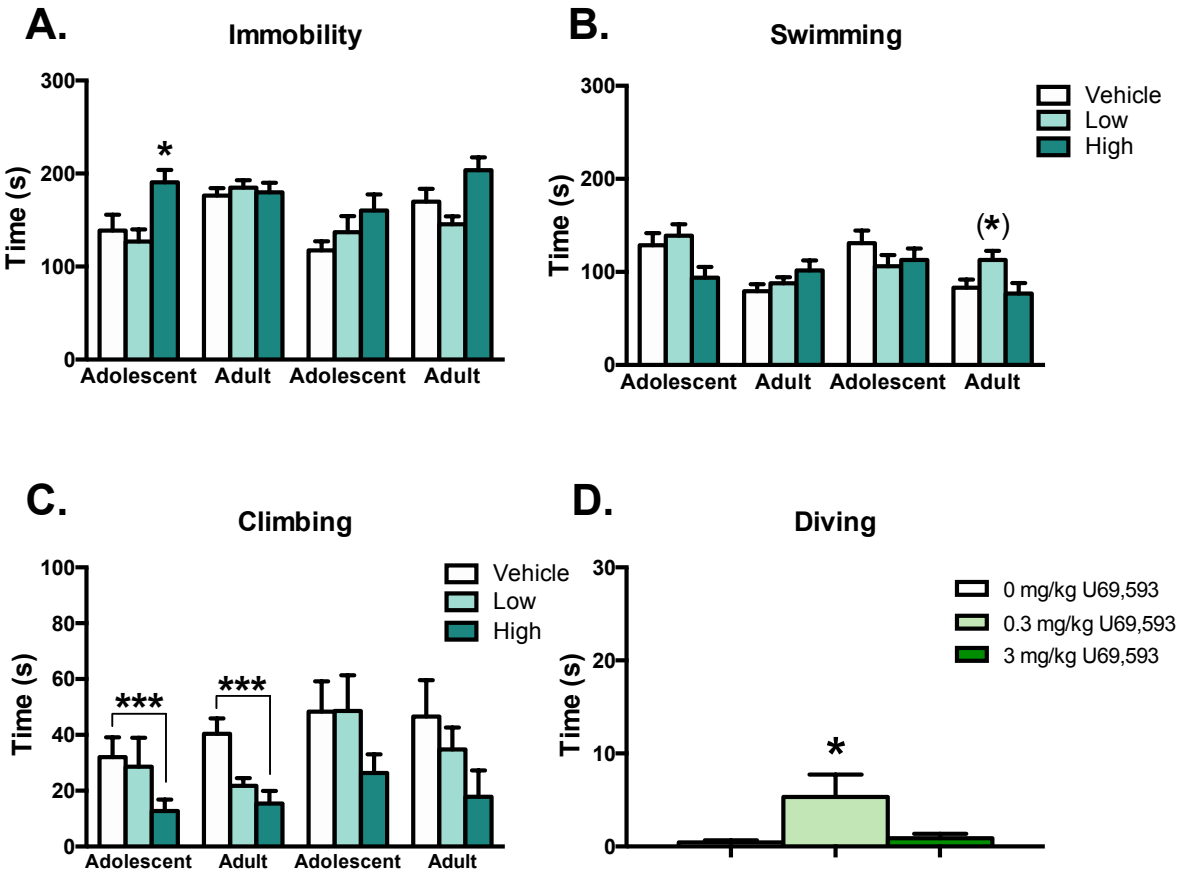

Figure 4.4. Adolescent males are more sensitive to the depressive-like effects of KOR activation in the FST than females or adult males. (A) Immobility was increased by high dose of KOR agonists compared to vehicle in adolescent males only. (B) Swimming was not significantly changed by KOR activation, but there was a trend for the low dose of KOR agonist to increase swimming compared to vehicle in adult females. (C) Climbing is decreased by high dose of KOR agonists compared to vehicle in adolescent and adult males. (D) Diving is increased by low doses of U69,593, but not U50,488, in all groups. Data represent mean + SEM. *, $p<0.05$; ***, $p<0.001$, (*), $p<0.1$ vs. vehicle. n = 11-17/group.

Discussion

In addition to its role in regulating the acute and chronic effects of nicotine and alcohol exposure (Chapter 3; Wee & Koob, 2010), a large body of literature links the dynorphin/KOR system to modulation of mood-associated behaviors, including anxiety and depression (Tejeda *et al.*, 2012b; Van't Veer & Carlezon, 2013). However, the

contribution of age and sex in modulating this interaction is poorly understood. Using a battery of well-established behavioral measures of anxiety- and depressive-like behavior, I have shown that pharmacological activation of KORs with U50,488 and U69,593 differentially modulates mood-associated behaviors based on age, sex, and behavioral task. Whereas males only displayed KOR-mediated anxiety-like behavior in the EPM, adult females showed increased anxiety in both LDT and EPM. Adolescent males were the only group to display significant depressive-like responses to KOR activation, while adolescent females were impervious to KOR activation across all tasks. Coupled with my prior findings (Chapter 3), these studies emphasize the complex, sex-dependent maturation of KOR function across development.

Anxiety-like behavior

Previous work has shown that KOR activity produces dysphoria, aversion, and anxiety (Pfeiffer *et al.*, 1986; Van't Veer & Carlezon, 2013). However, behavioral outcomes differ depending on sex and age. Females are generally considered to be less sensitive to the aversive, anxiogenic effects of KOR stimulation (Chartoff & Mavrikaki, 2015). Adolescent males, in contrast, have shown anxiogenic (Tejeda *et al.*, 2012a) and anxiolytic (Privette & Terrian, 1995; Braida *et al.*, 2009; Alexeeva *et al.*, 2012) responses to KOR activation. Previous work has also shown that acute Nic+EtOH exerts a KOR-dependent anxiolytic effect in adult females (Chapter 3), which suggests that KOR-mediated modulation of mood-associated behaviors differs across age and sex.

Using two common measures of anxiety-like behavior in drug naïve animals, I extend these findings to show that behavior in the LDT and EPM is differentially regulated by KOR based on age, sex, and measure. In males, KOR activation had no significant effect on anxiety in the LDT at any dose. This was somewhat unexpected, given the well-established role of KORs in anxiogenic responses (Van't Veer & Carlezon, 2013). It is important to note that these conclusions have been established largely through use of EPM. The LDT is rarely used, and data on agonist-induced anxiety using this test are mixed (Narita *et al.*, 2006; Wittmann *et al.*, 2009). However, my findings highlight the complexity of modeling anxiety in rodent models, and add to the literature suggesting that the LDT measures a different aspect of anxiety from the EPM (Steimer, 2011), or that this test is not ideal for assessing KOR-mediated anxiety-like behaviors in male rats. In contrast, KOR activation produced a robust increase in the time males spent in the closed arms of the EPM, suggesting increased anxiety. This was seen in both adolescent and adult rats at the high dose, which was unexpected given that other groups have reported anxiolytic effects of KOR activation in young male rats (Privette & Terrian, 1995; Alexeeva *et al.*, 2012), but may be explained by methodological differences, such as specific age of testing, handling, or drug administration procedure.

In females, the high dose of KOR agonist significantly increased anxiety-like behavior in the LDT and EPM for adult females. Adult female rodents are reportedly less sensitive to the effects of KOR activation than male rodents (Chartoff & Mavrikaki, 2015), but whether anxiety-like behavior is differentially affected by KOR agonists in females has not been examined until now. These findings suggest that adult females

are *more* sensitive to the anxiogenic effects of direct KOR activation. This was of particular interest given my prior finding that acute Nic+EtOH produced a KOR-dependent anxiolytic effect in adult females, and suggests that distinct circuitry mediates the effects of direct versus indirect KOR activation on anxiety. Indeed, high doses of KOR agonists used in the present study can be expected to robustly activate a large pool/distribution of receptors in multiple brain regions to drive anxiety. In contrast, the low dose of Nic+EtOH used likely produces minimal dynorphin release and low levels of KOR activation, which has been found to be anxiolytic (Privette & Terrian, 1995), and may activate KORs in a more restricted anatomical fashion.

Adolescent females, in contrast, were insensitive to the anxiogenic effects of KOR activation in either test and similarly insensitive to the anxiolytic effects of Nic+EtOH observed in adult females (Chapter 3). Although adolescent females may not have reached sexual maturity (Varlinskaya *et al.*, 2013), the observed age difference is unlikely to be mediated by variation in circulating ovarian hormones. Although some aspects of KOR function are influenced by estradiol (Abraham *et al.*, 2018), the aversive and negative affective components of KOR activity appear to be estradiol-independent (Russell *et al.*, 2014; Ehrich *et al.*, 2015; Abraham *et al.*, 2018; Conway *et al.*, 2019). Instead, these findings may be due to maturational changes within the BLA. The BLA is actively maturing during adolescence (Cunningham *et al.*, 2002; Rubinow & Juraska, 2009) and is a key locus for the dynorphin/KOR system's involvement in aversion and anxiety (Knoll *et al.*, 2011; Janak & Tye, 2015; Przybysz *et al.*, 2017). Recent work suggests that BLA KORs undergo a developmental shift in function, where KORs potentiate GABAergic transmission to inhibit anxiety during adolescence, but not

adulthood (Przybysz *et al.*, 2017). Although those findings were in males, I found female-specific age differences in KOR expression and function within the BLA (Chapter 3) that may help explain my present behavioral findings. Indeed, although adolescent females had higher KOR mRNA expression in the BLA, adult females had greater U69,593-stimulated [^{35}S]GTPγS binding, a measure of KOR function, than males and adolescent females (Chapter 3). The latter results may indicate that adult females are more sensitive to KOR activation in the BLA than younger females, ultimately resulting in increased anxiety-like behavior.

Although unlikely, pharmacokinetic differences could also contribute to the observed age and sex differences. Recent work demonstrated that adult female rats had significantly higher plasma levels of U50,488, as well as lower clearance, than males, which could suggest higher bioavailability for the agonist. However, they also reported similar brain levels of U50,488 and terminal half-life between males and females (Russell *et al.*, 2014).

Depressive-like behavior

Similar to what is observed when assessing anxiety-like behaviors, selective KOR agonists have been found to increase brain stimulation thresholds and immobility in the FST, both considered indicative of a depressive state (Mague *et al.*, 2003; Todtenkopf *et al.*, 2004; Russell *et al.*, 2014). Conversely, KOR blockade by antagonists has anti-depressant effects in non-stressed animals and during withdrawal from chronic drug exposure (Mague *et al.*, 2003; Carlezon *et al.*, 2006; Jarman *et al.*, 2018; Laman-

Maharg *et al.*, 2018), with females showing lower sensitivity to drug treatment in both paradigms (Russell *et al.*, 2014; Laman-Maharg *et al.*, 2018).

In order to examine whether adolescents and females exhibited differential sensitivity to the depressive-like effects of KOR activation, as they did anxiety-like effects, I used the FST. In line with human clinical research demonstrating higher rates of depression in women, many rodent studies show higher FST immobility in females compared to males (Drossopoulou *et al.*, 2004; Dalla *et al.*, 2008; Pitychoutis *et al.*, 2009). I did not observe significant age or sex differences in active or inactive coping behaviors of control animals, which may be due to the duration of handling prior to testing. Extending my findings of age- and sex-dependent differences in KOR-mediated regulation of anxiety-like behavior, I now show that KOR agonists did not have significant depressive-like effects in females and adult males, but increased immobility in adolescent males. This indicates an unexpected sensitivity to KOR's pro-depressant effects in adolescent males given prior data showing antidepressant effects of the KOR agonist Salvinorin A in young adult male rats (Braida *et al.*, 2009). At the same time, climbing was decreased by the high dose of KOR agonists in males of both ages, which suggests that KOR activation induced a shift from active to inactive (i.e., depressive-like) coping behaviors in adolescent males. When considered with my EPM results and evidence for both nicotine- and ethanol-induced dynorphin release (Lam *et al.*, 2008; Isola *et al.*, 2009), my data emphasize complex regulation of mood- and drug-associated behaviors by the dynorphin/KOR system. That is, whereas KOR signaling in adolescent males drives anxiogenic, pro-depressant effects and inhibits ethanol

reinforcement - likely via distinct circuitry - they lack the mature, inhibitory influence of KOR activity on Nic+EtOH reinforcement (Chapter 3; Lárraga *et al.*, 2017).

It is of particular interest that adolescent females appeared impervious to the effects of KOR activation in all three behavioral tasks. Along with prior findings that norBNI does not significantly alter nicotine and/or alcohol-mediated reinforcement or anxiety in adolescent females (Chapter 3; Lárraga *et al.*, 2017), these data suggest that KORs do not exert a strong modulatory influence over mood- or acute drug-associated behaviors during this developmental period. This insensitivity is unlikely to be due to age- and sex-specific differences in KOR density (Chapter 3), but may be due to immature interactions between corticotropin releasing factor (CRF) and dynorphin/KOR signaling (Bruchas *et al.*, 2009; Grella *et al.*, 2014). Future studies are needed to identify the specific mechanisms mediating adolescent females' unique immaturity in KOR function.

Conclusion

The present findings add to the growing body of literature highlighting the complex nature of KOR-mediated regulation of mood-associated behaviors. Although it is generally accepted that females and adolescents are less sensitive to the effects of KOR activation, my data suggest that this distinction is not clear cut for anxiety- and depressive-like behaviors. Instead, interactions between KOR signaling and these mood-associated behaviors depend on age, sex, measure, and behavioral state (e.g., drug naïve, non-stressed). Adolescent rats, in particular, exhibit a profound sex-dependent divergence in KOR function. Developmental and sex differences in KOR

sensitivity may modulate acute behavioral responses to common drugs of abuse, such as nicotine and alcohol, in unique ways, and future studies are needed to more fully understand these interactions. However, my data highlight the complexity of the dynorphin/KOR system and emphasize the importance of including age and sex in both preclinical and clinical studies of mood- and drug-associated behaviors.

Chapter 5

Discussion

I. Significance

Alcohol and nicotine, either from tobacco or increasingly from e-cigarettes, are the two most commonly co-used drugs of abuse. High prevalence, substantial negative health consequences, and economic burden make dual use of nicotine and alcohol a major public health concern, particularly among teenagers and young adults. Despite decreases in overall drug use among adolescents, concurrent nicotine and alcohol use has increased over time (Daw *et al.*, 2013). Furthermore, the use of e-cigarettes containing nicotine has surpassed traditional tobacco cigarette consumption among adolescents (Miech *et al.*, 2015b; Jamal *et al.*, 2017), and is associated with increased alcohol consumption, binge drinking, and AUD diagnoses (McCabe *et al.*, 2017; Roberts *et al.*, 2018; Hefner *et al.*, 2019; Thrul *et al.*, 2019).

During adolescence, the brain undergoes a marked reorganization in areas critical for executive function, learning and memory, and reward processing (Spear, 2000; Yuan *et al.*, 2015). Although essential for the transition to adulthood, these rapid changes leave the brain uniquely vulnerable to the deleterious effects of drugs of abuse, including nicotine and alcohol. Neurobiological mechanisms mediating high rates of nicotine and alcohol use during adolescence are poorly understood, but developmental changes in neurotransmitter systems known to modulate dopamine and serotonin signaling, such as the dynorphin/KOR system, likely contribute. Increasing evidence

suggests that age and sex influence KOR-mediated regulation of mood- and drug-associated behaviors. Thus, I have tested the hypothesis that maturational and sex-dependent changes in the dynorphin/KOR system modulate nicotine and alcohol responses, as well as anxiety- and depressive-like behaviors. My work emphasizes the profound complexity of the dynorphin/KOR system in modulating drug- and mood-associated behaviors, and highlights important maturational and sex-dependent changes in KOR function.

II. KOR regulation of nicotine and alcohol reinforcement

A wealth of literature has linked the dynorphin/KOR system to the behavioral and neurochemical effects of chronic drug exposure and drug dependence (Wee & Koob, 2010; Anderson & Becker, 2017), but there has been minimal investigation into KOR modulation of the *acute* effects of nicotine and alcohol. Using an acquisition of self-administration task, our lab previously demonstrated that concurrent Nic+EtOH is more reinforcing in adolescent males than in females or adult males. KOR blockade with the long-acting antagonist norBNI enhanced Nic+EtOH reinforcement in adult males only (Lárraga *et al.*, 2017). This suggests that drug-induced dynorphin release and subsequent KOR activation inhibit Nic+EtOH reinforcement in adult males, but that this protective mechanism is sex-dependent and still developing during adolescence. Given the growing body of literature demonstrating unique effects of concurrent nicotine and alcohol exposure (Cross *et al.*, 2017), we tested whether KOR signaling exerts similar age- and sex-dependent modulation of the reinforcing properties of each drug individually.

Pretreatment with norBNI strongly facilitates ethanol reinforcement in adolescent males and adult females, which suggests that KOR activity tonically inhibits ethanol reinforcement in these groups. In contrast, KOR blockade does not significantly alter ethanol self-administration in adult males or adolescent females. Although these findings contrast with prior findings that females and adolescents are less sensitive to KOR activation (Anderson *et al.*, 2013; Anderson *et al.*, 2014), and that norBNI decreases oral ethanol intake in adult female rats (Morales *et al.*, 2014), this is the first study to examine initial reinforcing effects of ethanol in a self-administration acquisition paradigm.

Nicotine self-administration was also age- and sex-dependently influenced by KOR manipulation. In males, nicotine was reinforcing in both age groups and self-administration was not significantly altered by norBNI pretreatment, although reinforced responses at this low drug dose (7.5 µg/kg/inf.) were significantly higher in adolescents than adults, as we have previously shown (Gellner *et al.*, 2016). The observed lack of effect in males is consistent with prior findings (Liu & Jernigan, 2011). Nicotine self-administration was unaffected by norBNI pretreatment in adolescent females as well, but was significantly inhibited by KOR blockade in adult females, suggesting that KOR activity may play a role in driving the initial reinforcing properties of nicotine in adult females.

Although the molecular and neurochemical targets of nicotine and alcohol overlap substantially (Cross *et al.*, 2017; Klenowski & Tapper, 2018), our prior work (Lárraga *et al.*, 2017) and the present findings suggest that distinct, drug-specific anatomical pathways containing KORs are involved in modulating the reinforcing

properties of nicotine and/or alcohol. My findings complement epidemiological and clinical literature indicating differential effects of individual versus concurrent nicotine and alcohol use (Rose *et al.*, 2004; Weinberger *et al.*, 2013; Chiappetta *et al.*, 2014; Hitschfeld *et al.*, 2015; Weinberger *et al.*, 2015), and emphasize the importance of studying the unique properties of concurrent versus individual nicotine and alcohol exposure when assessing mechanisms underlying co-use.

III. Drug and KOR agonist effects on mood-associated behaviors

Stress and mood alterations are a major contributing factor to drug use and dependence. KORs play an important role in regulating motivational and emotional states, and are classically associated with aversion, dysphoria, and anxiety (Pfeiffer *et al.*, 1986; Van't Veer & Carlezon, 2013; Lalanne *et al.*, 2014), though important age and sex differences exist. Interactive effects of age, sex, and KOR signaling on mood-associated behaviors independently or in response to nicotine and/or alcohol exposure are poorly understood.

Nicotine and/or ethanol effects on anxiety-like behavior

The present studies demonstrate that KORs age- and sex-dependently modulate drug-induced anxiety-like behavior, as measured by time spent in the center of an open-field. Whereas adult males were not significantly affected by drug or norBNI pretreatment, concurrent Nic+EtOH may be anxiolytic in adolescent males. However, this effect is likely subtle, given that my initial finding of a significant Nic+EtOH-induced decrease in anxiety (Chapter 2) was not replicated in a subsequent study (Chapter 3).

This discrepancy may be due to the latter study being underpowered and requiring additional statistical comparisons that could have precluded statistical significance. General locomotion was also strongly stimulated by ethanol and Nic+EtOH in adolescent males, and the latter effect was blocked by norBNI pretreatment, indicating a KOR-dependent mechanism.

Although ethanol was anxiogenic for adolescent females, this was not mediated by KORs since receptor blockade had no effect (Chapter 3). In contrast, Nic+EtOH had significant anxiolytic effects in adult, but not adolescent, females that were blocked by norBNI. This was unexpected given literature linking anxiogenic and depressive effects with KOR activation (Van't Veer & Carlezon, 2013). However, the low dose of Nic+EtOH used here may have induced a small increase in dynorphin levels and minimal KOR activation compared to receptor activation following high doses of selective agonists. Indeed, low doses of KOR agonists (e.g., 100-1000 μg/kg) can be anxiolytic (Privette & Terrian, 1995). Furthermore, distinct circuitry likely regulates Nic+EtOH-induced versus direct KOR activation.

My data demonstrate important interactive effects of age and sex on KOR modulation of drug-induced anxiety that differ based on drug. Coupled with my finding that nicotine alone was without significant effect in any group tested, my data further emphasize the unique consequences of combined nicotine and alcohol compared to either drug alone.

KOR regulation of mood-associated behaviors

To explore whether direct activation of KORs also age- and sex-dependently modulated mood-associated behaviors, I used the LDT and EPM to test the hypothesis that adolescents and females would be less sensitive to the anxiogenic effects (Chartoff & Mavrikaki, 2015) of direct KOR activation by the selective agonists, U50,488H and U69,593 (Chapter 4). In general, I found that both KOR agonists produced similar behavioral responses, and data were collapsed across drug. KOR activation produced a robust increase in the time both adolescent and adult males spent in the closed arms of the EPM, indicating increased anxiety. Although other groups have reported anxiolytic effects of KOR agonists in young male rats (Privette & Terrian, 1995; Braida *et al.*, 2009; Alexeeva *et al.*, 2012), my finding of a lack of age difference may be explained by methodological differences, such as specific age of testing, handling, or drug administration procedure. In contrast to EPM, the KOR agonists did not induce anxiety in the LDT, emphasizing that different aspects of anxiety are measured in the two tests, as has been shown before (Steimer, 2011).

In adult females, the high dose of KOR agonist significantly increased anxiety-like behavior in both the LDT and EPM. This suggests that adult females are *more* sensitive than males to the anxiogenic effects of direct KOR activation in drug naïve, non-stressed states. This was of particular interest given my prior finding that acute Nic+EtOH produced a KOR-dependent anxiolytic effect in adult females, and suggests that distinct circuitry mediates direct versus indirect KOR activation on anxiety. Indeed, high doses of KOR agonists as used in the present study can be expected to robustly activate a large pool of receptors in multiple brain regions to drive anxiety. The low dose of Nic+EtOH, in contrast, likely only produces low levels of KOR activation, which has

been found to be anxiolytic (Privette & Terrian, 1995), and may activate KORs in a more restricted anatomical fashion. In contrast to males and adult females, adolescent females were resistant to the anxiogenic effect of KOR agonists in both tests of anxiety, suggesting late maturation of the relevant circuitry in females.

KOR activation has also been associated with depressive effects (Mague *et al.*, 2003; Todtenkopf *et al.*, 2004; Carlezon *et al.*, 2006; Russell *et al.*, 2014; Laman-Maharg *et al.*, 2018). Limited data exist examining the influence of sex and age on the pro-depressant effects of KOR signaling, though females have been reported to be less sensitive than males (Russell *et al.*, 2014; Laman-Maharg *et al.*, 2018). Using the FST, I have found that immobility was increased by KOR activation in adolescent males only, although climbing was decreased in all groups. Although prior data show antidepressant effects of the KOR agonist Salvinorin A in young adult male rats (Braida *et al.*, 2009), my data indicate an unexpected sensitivity to KOR's pro-depressant effects in adolescent males, and suggest that KOR activation induces a shift from active to inactive (i.e., depressive-like) coping behaviors in this group.

The overall insensitivity of adolescent females to KOR manipulation is of particular interest. U69,593 and U50,488H had no effect on any of the included behavioral measures for this group, and when coupled with prior findings that norBNI does not significantly alter nicotine and/or alcohol-mediated reinforcement or anxiety (Chapter 3; Lárraga *et al.*, 2017), these data suggest that KORs do not exert a strong modulatory influence over mood- or acute drug-associated behaviors in adolescent females.

III. Neuroanatomy

In order to identify possible neuroanatomical correlates of the observed age and sex differences in KOR-mediated regulation of drug- and mood-associated behaviors, I measured KOR mRNA expression and G-protein coupling in brain areas critical for reward processing and mood regulation. I found that adult females had significantly higher U69,593-stimulated [^{35}S]GTPγS binding, a measure of KOR function, in the PVH and amygdala compared to males or adolescent females, but this was not paralleled by increased KOR mRNA expression. The BLA, in particular, is a key locus for the dynorphin/KOR system's involvement in aversion and anxiety (Knoll *et al.*, 2011; Janak & Tye, 2015; Przybysz *et al.*, 2017), and is actively maturing during adolescence (Cunningham *et al.*, 2002; Rubinow & Juraska, 2009). Recent work shows that KORs potentiate GABAergic transmission to inhibit anxiety during adolescence, but not adulthood, which suggests that BLA KORs undergo a developmental shift in function to drive anxiety (Przybysz *et al.*, 2017). Thus, the increased BLA KOR function I observed may indicate that adult females are more sensitive to KOR activation in the BLA than males or younger females, ultimately resulting in increased anxiety-like behavior.

Analysis of regional neuronal activity and functional connectivity suggest that maturational changes in KOR-mediated regulation of nicotine and alcohol responses converge within the midbrain. Indeed, I demonstrated adult-specific alterations in regional *cFos* mRNA expression in limbic areas after Nic+EtOH treatment, and found that functional networks involving the pVTA are differentially recruited by Nic+EtOH to modulate reinforcement and anxiety-like behavior (Chapter 2). Protective mechanisms, such as glutamate projections from the BNST to non-dopaminergic neurons in the VTA

that drive aversion and block reward (Jennings *et al.*, 2013; Vranjkovic *et al.*, 2017), may be recruited by Nic+EtOH exposure in adults to increase pVTA cFos expression and inhibit reinforcement (Lárraga *et al.*, 2017), while they are not recruited in adolescence. Similarly, Nic+EtOH may recruit a dynorphin projection from the LH to the pVTA in adults that blocks DA release and drug reinforcement via a KOR-dependent mechanism. Future studies are needed to test these hypotheses, but my data highlight the complex maturation of limbic circuitry involved in regulating the acute effects of Nic+EtOH.

In contrast, my finding that KOR signaling in adolescent, but not adult, males drives anxiogenic, pro-depressant effects and inhibits ethanol reinforcement suggests that distinct - and poorly understood - circuitry modulates these interactions. Indeed, drug naïve adolescent males had higher KOR functional activity in the VTA and MR than adult males, as measured by [^{35}S]GTPγS binding. Very little is known about the function of KORs within the MR, and adolescent-specific KOR function within both regions has not been well studied. However, the MR sends glutamatergic projections to the VTA that negatively regulate dopamine transmission (Wirtshafter & Trifunovic, 1992; Geisler *et al.*, 2007; Geisler & Wise, 2008; Shim *et al.*, 2014), and microinjections of dynorphin into the MR stimulate consummatory behaviors (Klitenick & Wirtshafter, 1995). Thus, greater receptor function within these regions may result in a stronger influence over ethanol reinforcement and negative affective behaviors in adolescent males.

IV. Limitations and future directions

Gonadal hormones and KOR signaling

Estrous cycle was not measured in any of my studies. In addition to vaginal lavage producing a confounding stress effect, the numerous age and sex differences in KOR function are unlikely to be mediated by variation in circulating ovarian hormones. Although some aspects of KOR function, such as analgesia, are influenced by estradiol (Abraham *et al.*, 2018), the aversive and negative affective components of KOR activity have been shown to be unaffected by ovariectomy or stage of estrus cycle (Russell *et al.*, 2014; Ehrich *et al.*, 2015; Abraham *et al.*, 2018; Conway *et al.*, 2019).

Intracellular signaling cascades at the KOR

Increasing pharmacological evidence demonstrates biased KOR signaling that produces disparate effects on behavior. Whereas bias towards the G-protein pathway is involved in the antinociceptive and dopamine-inhibiting effects of KOR activation, β-arrestin recruitment and p38 MAPK activation mediates aversive and dysphoric effects (Bruchas *et al.*, 2007; Bruchas *et al.*, 2011; Chavkin *et al.*, 2014; Ehrich *et al.*, 2015; Abraham *et al.*, 2018). The [^{35}S]GTPγS binding assay used in the present study measures G-protein coupling and is unable to provide a measure of β-arrestin recruitment. This limits comparison between our U69,593-stimulated [^{35}S]GTPγS binding and behavioral data. However, we are the first to conduct direct age and sex comparisons of G-protein signaling at the KOR, and it is possible that similar developmental and sex differences exist in β-arrestin biased signaling, although future studies are needed to test this.

Region-specific KOR signaling

The present study demonstrated that regional KOR mRNA expression and receptor function differ across development and between sex. Greater KOR function in stress-associated regions, such as the BLA, may underlie adult females' sensitivity to the anxiogenic effects of selective KOR agonists. Future studies using BLA-specific injections of KOR antagonists would provide better anatomical specificity for explaining the anxiety and reinforcement alerting effects, respectively.

I also showed that, in males, acute Nic+EtOH produces adult-specific alterations in regional neuronal activity and functional connectivity that may exert a protective, inhibitory influence on Nic+EtOH reinforcement. One potential mechanism is drug-induced activation of LH dynorphin inputs to pVTA dopamine neurons, where KOR activation would inhibit dopamine neuron activity to block reward. Future experiments to assess this hypothesis could include systemic and intra-pVTA norBNI pretreatment to test whether Nic+EtOH effects on neuronal activity and CGE are blocked. Chemogenetic targeting of LH-VTA dynorphin projections, coupled with intra-pVTA norBNI, in animals self-administering Nic+EtOH could also be used to examine the pathway-specificity.

V. Overall conclusions

Neurobiological mechanisms underlying high rates of nicotine and alcohol co-use are not well understood. Although the dynorphin/KOR system has classically been associated with chronic drug exposure and dependence (Wee & Koob, 2008), my

dissertation highlights maturational and sex-dependent changes in KOR function that modulate sensitivity to the acute behavioral effects of nicotine and alcohol. Using behavioral pharmacology and anatomical approaches, I have demonstrated that whereas KOR activity appears to tonically inhibit ethanol reinforcement in adolescent males and adult females, it may play a role in driving the initial reinforcing properties of nicotine in adult females. These effects are unique to these groups and are distinct from KOR modulation of concurrent Nic+EtOH (Lárraga *et al.*, 2017). Anatomical studies suggest that protective mechanisms, such as glutamate and/or dynorphin inputs to the VTA that drive aversion and block reward, may be differentially recruited in adolescence versus adulthood to modulate drug reinforcement. Despite molecular and neurochemical targets of nicotine and alcohol overlapping substantially, my findings suggest that distinct, drug-specific anatomical pathways containing KORs are involved in modulating the reinforcing properties of nicotine and/or alcohol.

I have also demonstrated that mood-associated behaviors are differentially regulated by indirect (i.e., drug-induced) versus direct (i.e., selective agonists) KOR activation. Nic+EtOH exerted a robust, KOR-dependent anxiolytic effect in adult females that was paralleled by increased KOR function in the BLA. This was of particular interest given my finding that treatment with the selective KOR agonists, U50,488H and U69,593, produced a robust increase in anxiety-like behavior in adult females, but may suggest that distinct circuitry mediates direct versus indirect KOR activation on anxiety. Although Nic+EtOH may produce a subtle anxiolytic effect in adolescent males, both adolescent and adult males displayed increased anxiety in the EPM following high dose KOR agonist treatment. Adolescent males were also sensitive to the pro-depressant

effects of KOR activation, while other groups were not. Some research has shown anxiolytic effects of KOR agonists in young male rats (Privette & Terrian, 1995; Braida *et al.*, 2009; Alexeeva *et al.*, 2012), but these data highlight the complexity of KOR maturation. Adolescent females were largely impervious to the effects of KOR manipulation on mood- or drug-associated behaviors, highlighting a unique and previously unknown immaturity of KORs in this group. As a whole, my work emphasizes the profound complexity of KOR signaling and highlights important maturational and sex-dependent changes in KOR function. These changes may act as a critical, but underrecognized, influence of nicotine and alcohol co-use among teenagers and women.

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