INFLUENCE OF ADOLESCENT SOCIAL INSTABILITY STRESS ON THE INTAKE OF ETHANOL AND SUCROSE IN A RODENT MODEL

by
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Abstract

Adolescence is a sensitive period in which the effects of stress and alcohol can have long-lasting impacts. Social instability stress in adolescent rats (SS; postnatal day 30-45, daily 1 hour isolation + new cage partner) alters behavioural responses to psychostimulants and increases anxiety-like behaviour, but differences in voluntary consumption of natural and drug rewards are unknown. The main goal of my thesis was to investigate the effects of adolescent social instability stress (SS) on immediate and long-lasting changes on reward-related behaviours in male rats using voluntary alcohol intake paradigms. Another goal was to investigate the influence of social context on the propensity to drink alcohol, as well as the influence of these factors on sucrose intake. In chapter 2, I found that adolescent SS increased alcohol intake irrespective of social context, and adolescents drank more alcohol than adults. The intake of sucrose was not altered by stress, except during context of competition. In chapter 3, I found that history of alcohol drinking reduced synaptic plasticity markers in the dorsal hippocampus and prefrontal cortex, and this reduction was sometimes further reduced by SS. The propensity to drink alcohol was found not to differ between SS and CTL rats in the first experiment, and reduced among SS rats in the second experiment. After nine days of alcohol absence, the propensity to drink alcohol was not increased by previous alcohol access, and SS increased intake only in alcohol-naïve rats. History of alcohol drinking reduced anxiety-like behaviours and blunted SS-induced reduction in social interactions. Both SS and alcohol decreased corticosterone levels at baseline and after fear recall without changing freezing behaviour. My findings indicate that using a model of mild social stressor can have great impact on adolescent rats, but moderate effects in adult rats. The behavioural changes caused by stress can be
enhanced later in life by history of alcohol drinking, but that does not necessarily cause an increase in the propensity to drink during adulthood, as other studies have shown. Adolescent stressed rats drink more alcohol than other groups, but they don’t seem to continue drinking more when they reach adulthood. These results indicate that the effects of social instability stress are transient in regards to propensity to drink, and can be the basis for alterations caused by both alcohol and stress.

**Keywords:** adolescence, social instability stress, alcohol, sucrose, drinking, reward
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropin hormone</td>
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<tr>
<td>BAC</td>
<td>Blood alcohol content</td>
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<tr>
<td>BW</td>
<td>Body weight</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Calcium/calmodulin-dependent kinase II</td>
</tr>
<tr>
<td>CTL</td>
<td>Non-stressed control group</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotropin releasing hormone</td>
</tr>
<tr>
<td>D1</td>
<td>Dopamine receptor 1</td>
</tr>
<tr>
<td>D2</td>
<td>Dopamine receptor 2</td>
</tr>
<tr>
<td>DH</td>
<td>Dorsal hippocampus</td>
</tr>
<tr>
<td>EPM</td>
<td>Elevated Plus Maze</td>
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<tr>
<td>EtOH</td>
<td>Ethanol</td>
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<td>GABA</td>
<td>γ-aminobutyric acid</td>
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<td>GR</td>
<td>Glucocorticoid receptor</td>
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<tr>
<td>HPA</td>
<td>Hypothalamus-hypophysis-adrenal axis</td>
</tr>
<tr>
<td>IA2BC</td>
<td>Intermittent access to 2-bottle choice model</td>
</tr>
<tr>
<td>MR</td>
<td>Mineralocorticoid receptor</td>
</tr>
<tr>
<td>MSN</td>
<td>Medium spiny neurons</td>
</tr>
<tr>
<td>nAcc</td>
<td>Nucleus accumbens</td>
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<tr>
<td>PFC</td>
<td>Prefrontal cortex</td>
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<tr>
<td>PND</td>
<td>Postnatal day</td>
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<td>Paraventricular nucleus of the hypothalamus</td>
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<td>PSD-95</td>
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<td>Social instability stress model/group</td>
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<td>VH</td>
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Preface

The adolescent period has been recognized as a critical period of development, in which brain systems are still maturing and shaping the future adult individual. Because of the intense brain maturation happening during adolescence, this period of life is particularly vulnerable to life events such as exposure to stressor and drugs of abuse which can have long-lasting effects. The main goal of my thesis was to investigate the effects of social stress during the adolescent period on reward-related behaviours, testing whether stressed rats would consume more rewarding substances such as ethanol and sucrose, and whether they would be more vulnerable to negative effects of ethanol later in life than non-stressed rats. In the first chapter, I describe the brain structures involved in responses to rewards and how alcohol activates this system. I then describe how the stress response system works and how it can influence alcohol intake. Later, I define the adolescent period, identify which behaviours are found to be unique to this period, and describe how the reward and stress systems are maturing during this period. Further, I review the literature regarding adolescence ethanol exposure in rodents, which models have been used and how can adolescence stress influence alcohol intake. Finally, I explain the stress model used in this thesis, what is been known about it, and describe the goals of my thesis. In the second chapter, I investigated how adolescent social instability stress affect the consumption of ethanol and sucrose in adolescent and adult rats in a variety of social contexts. In the third chapter, I investigated the long-lasting effects of social instability stress on the propensity to drink ethanol and negative consequences in terms of anxiety, corticosterone concentrations and expression of key proteins and receptors involved in stress and reward systems. In the fourth chapter, I discuss my findings, their implications and limitations, as well as future perspectives for my research.
Chapter 1: General Introduction

Given the world-wide popularity of alcohol, the frequent use and potential for use disorders of this substance, and the resulting enormous cost to society (Peacock et al., 2018), understanding how alcohol affects our brain remains a high priority. Alcohol is a complex drug from a pharmacological standpoint. It directly interacts with a large number of molecular targets (Abrahao, Salinas, & Lovinger, 2017), and indirectly influences perhaps an even larger number of biochemical reactions (Vengeliene, Bilbao, Molander, & Spanagel, 2008). These effects are also dose, timing, and exposure regimen dependent (Vengeliene et al., 2008). Research in animal models has contributed to uncovering new therapeutic potentials to reduce the negative consequences of alcohol on brain structure and function (Cannella et al., 2019; Farokhnia, Faulkner, Piacentino, Lee, & Leggio, 2019). Such studies have been conducted primarily in adult animals, with little known about the potential moderating effects of developmental stage for such therapeutics. Exposure during the early developmental periods is particularly important because alcohol can influence the development of the brain, and result in more pervasive and long-lasting effects than the same exposure in adulthood. Thus, understanding how acute or chronic alcohol exposure alters brain function is not an easy task, and despite decades of research of these questions, much remains to be discovered.

Since the goal of this thesis was to investigate the effects of adolescent social stress on alcohol intake, I will start by describing the system involved in reward responses.
**Reward system**

The reward system is a collection of brain structures that are responsible for the detection of pleasurable stimuli, promote motivation or desire for that stimulus, associative learning and reinforcement of behaviours that allow for the consumption of the stimuli again (reviewed in Schultz, 2015). The mesocorticolimbic dopamine system has been implicated as the major neurotransmitter system involved in reward-related behaviours (reviewed in Salamone & Correa, 2012). Researchers have identified many structures that process this type of rewarding information, but the major ones are the ventral tegmental area (VTA), nucleus accumbens (nAcc, which is part of the ventral striatum), dorsal striatum, prefrontal cortex (PFC), alongside the hippocampus and basolateral amygdala (reviewed in Koob & Volkow, 2010). Figure 1-1 illustrates the aforementioned brain structures.

![Sagittal view of the rat brain](http://labs.gaidi.ca/rat-brain-atlas/)

**Figure 1-1.** Sagittal view of the rat brain (0.9 mm lateral). The colored areas indicate four structures that are part of the reward system: prefrontal cortex (orange), nucleus accumbens (green), ventral tegmental area (red) and hippocampus (blue). The areas in grey indicate the structures are part of the stress response system, paraventricular nucleus of the hypothalamus and the pituitary, that will be mentioned in more details later. The medial view does not allow to see the basolateral amygdala and the dorsal striatum clearly. Image adapted from [http://labs.gaidi.ca/rat-brain-atlas/](http://labs.gaidi.ca/rat-brain-atlas/)
The VTA is a heterogeneous structure with no defining borders (not a nucleus) situated in the midbrain near the substantia nigra. Its physiological function was largely ignored until the discovery that it contains dopaminergic neurons (the A10 group) and that these neurons project widely to limbic areas implicated in motivation and positive reinforcement and to the selection, initiation, and invigoration of learned appetitive behaviors (reviewed in Fields, Hjelmstad, Margolis, & Nicola, 2007). Although commonly identified as a dopaminergic region, less than 60% of VTA neurons are dopaminergic, and the majority of the neurons are GABAergic (produce \(\gamma\)-aminobutyric acid, GABA) (Margolis, Lock, Hjelmstad, & Fields, 2006). VTA neurons can also be glutamatergic, and some neurons can co-release dopamine and GABA, dopamine and glutamate, or GABA and glutamate. VTA neurons can also synthesize neuropeptides (e.g., parvalbumin, cholecystokinin, corticotropin releasing hormone), and express receptors for other neuropeptides, such as insulin, orexin, melanocortin, and leptin. The electrophysiological properties of these neurons are also very complex, and their activity can be modified in response to different neurotransmitters. For example, dopaminergic neurons can be inhibited by the activation of opioid and GABA\(_B\) receptors, whereas glutamatergic neurons can be activated by dopamine receptor type 2. Dopaminergic neurons are identified by the presence of tyrosine hydroxylase (TH), the first enzyme involved in the synthesis dopamine, and of vesicular monoamine transporter 2 (VMAT2), which pumps dopamine into synaptic vesicles. GABAergic neurons are identified by the presence of glutamate decarboxylase (GAD), the enzyme that produces GABA from glutamate, and vesicular GABA transporters (VGAT). Glutamatergic neurons are identified by presence of vesicular glutamate transporters (VGLUT) (reviewed in Morales & Margolis, 2017). The activation of VTA dopamine neurons usually
promotes reward-seeking behaviours, whereas the activation of VTA GABAergic neurons disrupts these behaviours (Van Zessen, Phillips, Budygin, & Stuber, 2012) (in more details below).

The VTA receives GABAergic and glutamatergic projections from many brain structures such as the nAcc, PFC, lateral habenula, lateral hypothalamus, periaqueductal gray, bed nucleus of the stria terminalis, dorsal raphe, other tegmental areas and from local GABAergic, dopaminergic and glutamatergic neurons within the VTA. The projections sent from the VTA target the nAcc, PFC, amygdala, lateral habenula, periaqueductal gray, bed nucleus of the stria terminalis, hippocampus, ventral pallidum, locus coeruleus and the olfactory tubercle. The VTA dopaminergic projections that target the nAcc are called the mesolimbic pathway, and the ones that target the PFC are called the mesocortical pathway, and together they are called the mesocorticolimbic pathway (Björklund & Dunnett, 2007; Lammel et al., 2008). These dopaminergic projections are of great interest in the studies of reward and reinforcement since they were characterized in the late 1970s (see next section for details). However, VTA projections to these are more complex than simply dopaminergic neurons. The projections to the PFC co-release dopamine and glutamate, whereas the projections to the nAcc can be solely dopaminergic, GABAergic, glutamatergic, or co-release dopamine and GABA, or dopamine and glutamate. The majority of dopaminergic outputs from the VTA target the medium spiny neurons of the nAcc (Morales & Margolis, 2017). An schematic representation of the VTA outputs and inputs to the PFC and nAcc can be seen in Figure 1-2.
Figure 1-2. Schematic representation of the connections between the VTA with the PFC and nAcc.

The nAcc is part of the ventral striatum localized in the base of the telencephalon. It receives dopaminergic, glutamatergic and GABAergic inputs from the VTA, PFC, hippocampus, amygdala, thalamus and substantia nigra (reviewed in Salgado & Kaplitt, 2015). The nAcc is formed mainly by GABAergic medium spiny neurons (MSN) that can be divided in two types, depending on the dopamine receptor present: D1 and/or D2 (Scofield et al., 2016). Both dopamine receptors are G-coupled protein metabotropic receptors; D1 receptors are associated with Ga\textsubscript{s} subunits, which activate adenylyl cyclase, promote the production of cyclic adenosine monophosphate (cAMP) and activate signalling pathways associated with protein kinase A (PKA), whereas D2 receptors are associated with Ga\textsubscript{i} subunits, which have the opposite effects.
and inhibit cAMP production (Beaulieu & Gainetdinov, 2011). Therefore, these receptors have opposite effects on medium spiny neurons, and D1-MSN seem to positively regulate reward-related behaviours, whereas D2-MSN seems to negatively regulate these behaviours (Lobo et al., 2010). These neurons send projections to the basal ganglia and the ventral pallidum, which sends projections to the thalamus that projects to the PFC; the nAcc also sends projections back to the substantia nigra and VTA directly or indirectly via ventral pallidum, as well as to the bed nucleus of the stria terminalis, hypothalamus, lateral septum and habenula (Salgado & Kaplitt, 2015). The projections from the nAcc do not follow the same pattern as the dorsal striatum, in which D1-MSN are part of the “direct pathway” and send projections to the basal ganglia, whereas D2-MSN send projections first to the ventral pallidum and then to the basal ganglia, forming the “indirect pathway”. Instead, nAcc D1- and D2-MSN both send projections to the ventral pallidum, and some D2-MSN send projections directly to the thalamus (Klawonn & Malenka, 2019; Kupchik et al., 2015). The nAcc medium spiny neurons can also be divided into patch or matrix compartments, in which patches have dense µ-opioid receptor-binding sites, whereas matrices have less opioid binding and more acetylcholinesterase activity (Salgado & Kaplitt, 2015).

The nAcc can be divided into two main parts: the core and the shell. The core has a high density of dendritic spines and branches compared to the shell and projects mainly to the basal ganglia, whereas the shell projects to other regions as well (Scofield et al., 2016). The core acts as an intermediate between the shell and the dorsal striatum, and it is responsible for acquisition of reward-seeking behaviours. Dopamine antagonists injected into the core disrupt general Pavlovian conditioning, in which a cue is paired with the presentation of a reward (food) and elicits lever presses to obtain another reward (reviewed in Salamone et al., 2016). On the other
hand, the shell is involved with motivation and functions as a “hedonic hot spot” mediating the incentive salience of rewards (Berridge & Kringelbach, 2015; Berridge, Robinson, & Aldridge, 2009). For example, injection of dopamine antagonists into the shell, but not the core, prevents the self-administration of drugs such as cocaine in rats (Scofield et al., 2016).

**History of reward studies**

In 1954, Olds and Milner (Olds & Milner, 1954) discovered that rats would press a bar many times in order to obtain electrical stimulation in certain areas of the midbrain, indicating the existence of a brain reward system (reviewed in Koob, 2014). In the 1970s, evidence emerged that the brain regions that activated reward responses from the electrical stimulation studies were in fact dopaminergic neurons, and they responded to drugs of abuse: cocaine self-administration in rats was abolished when dopaminergic-responsive neurons in the nAcc were ablated, but not when noradrenergic-responsive neurons were ablated (Roberts, Corcoran, & Fibiger, 1977). Subsequently, a systematic characterization using microdialysis to measure dopamine release after systemic administrations of amphetamine, cocaine, morphine, methadone, ethanol, and nicotine showed strong increases of dopamine for all these substances (Di Chiara & Imperato, 1988). Later, in the 1990s, it was discovered that when rats were trained to receive a reward after a conditioned stimulus (CS) occurred, dopamine was released not when the reward was given (like during the first time the CS pairing happened) but immediately after presenting the CS. This finding indicated that dopamine bursts act as reward predicting signals. When the prediction of the reward did not happen (CS was not followed by a reward), dopamine release was depressed at the time the reward was expected (reviewed in Schultz, 1998). This discovery led to the study of dopamine release as a crucial aspect of the learning process involved in drug
abuse. The current hypothesis is that the increase in dopamine release in the mesocorticolimbic system induced by drugs of abuse could promote changes in the synaptic plasticity of these structures, altering their function and inducing drug-related behaviours that could lead to addiction (reviewed in Berke, 2018; Lüscher, 2016).

**Activation of the reward system by “natural” rewards**

VTA dopaminergic neurons have two modes of transmission, a tonic (steady baseline firing) and a phasic mode (quick bursts of neuronal activity) (Schultz, 2007). The phasic dopamine firing is triggered in the VTA by rewards or cues predicting rewards, which can trigger motivation and reinforcement of a reward-seeking behaviour. Optogenetic stimulation producing bursting patterns, but not tonic patterns, on VTA dopamine neurons induce conditioned place preference, which is a commonly used test to assess reward-seeking behaviour (Tsai et al., 2009). When an unexpected reward is encountered, VTA neurons are activated and release dopamine, and when this encounter happens repeatedly, these neurons stop being activated by the reward presentation but rather by a cue that precedes the reward (Schultz, 2015). Therefore, dopamine neurons are thought to mediate reward prediction error, reporting differences between the reward that is received and the reward that is predicted to occur. When a reward is better than predicted, dopamine neurons are activated (positive prediction error); when a reward is worse than predicted or fails to happen, dopamine neurons are inhibited (negative prediction error); and when a reward happens as expected, dopamine neurons have no response (zero prediction error) (reviewed in Bromberg-Martin, Matsumoto, & Hikosaka, 2010).
Activation of the reward system by drugs of abuse

Drugs of abuse can increase dopamine release from the VTA by different modes of action. Some drugs can directly depolarize dopaminergic neurons (such as nicotine), others inhibit dopamine reuptake in the synaptic cleft (like amphetamine, cocaine and ecstasy), and others hyperpolarize GABAergic neurons and disinhibit dopaminergic neurons (such as opioids, cannabis, benzodiazepines) (reviewed in Lüscher, 2016). Ethanol can act directly on VTA neurons by either increasing excitation and decreasing inhibition of different neurotransmitter systems (GABA, glutamate, glycine, acetylcholine) (reviewed in You, Vandegrift, & Brodie, 2018). With chronic drug use or drug abuse, the reward system goes through changes that allow the development of addiction. For example, chronic exposure to a wide variety of abused drugs upregulates cAMP formation, cAMP-dependent PKA activity, and PKA-dependent protein phosphorylation in the nAcc. Activation of PKA can mediate activation of the ERK pathway and modulate dendritic spine restructuring, transport of AMPA receptors to synapses, and increase neuronal excitability, which can promote drug-seeking behaviours (reviewed in Koob & Volkow, 2016). There is also accumulation of ΔFosB, a gene transcription factor, in nAcc D1-MSN after repeated drug use, which is implicated in drug sensitization and increased self-administration (Pitchers et al., 2013).

Alcohol and the reward system

Whereas many drugs of abuse have one specific target or mechanism of action in the brain, alcohol has many targets and affects many neurotransmitter systems (reviewed in Lovinger, 2018). One of the most studied is the GABAergic system, and alcohol has different effects depending on the composition of subunits’ of the GABAA receptors. It has been reported
that the δ subunit, therefore the brain regions with more expression of δ subunit, is more sensitive to alcohol’s effects than other subunits (reviewed in Tabakoff & Hoffman, 2013). The effects of alcohol on the VTA seem to be indirect, by decreasing neuronal activity of GABAergic interneurons and allowing an increase in dopamine release from the VTA (reviewed in Abrahao, Salinas, & Lovinger, 2017).

When absorbed by the gastrointestinal tract, alcohol enters the blood stream and can reach the brain. Ethanol is a small amphiphilic molecule, and can cross the blood-brain-barrier easily without the need of a transporter. To monitor and better understand the effects of alcohol in the body, researchers measure the blood alcohol content (BAC) of intoxicated individuals. BAC can vary depending on the concentration of alcohol ingested (%), the dose (g/kg of body weight) and the amount of water consumed with alcohol (volume) (Dilley, Nicholson, Fischer, Zimmer, & Froehlich, 2018). The age of the organism being tested also influences the BAC, and adolescent rats usually have lower BAC compared to adults when given the same dose by body weight (Walker & Ehlers, 2009). High levels of BAC (> 100 mg/dl) can be detected after 30 minutes of ingestion, and levels decrease with metabolization after 1 hour, although BAC can remain high for hours when high concentrations or doses of alcohol are being consumed (Dilley et al., 2018).

**Alcohol addiction cycle**

The addiction to alcohol, or alcoholism, can be defined as a chronically relapsing disorder characterized by (i) compulsion to seek and take the drug (alcohol), (ii) loss of control in limiting (alcohol) intake, and (iii) emergence of a negative emotional state (e.g., dysphoria, anxiety and irritability) reflecting a motivational withdrawal syndrome when access to the drug
(alcohol) is prevented, which is defined as drug dependence. Alcoholism takes place in three recurring stages: (i) initial intoxication, usually in the form of binge drinking, (ii) negative effects caused by alcohol withdrawal, and (iii) relapse due to preoccupation and anticipation from the negative effects, repeating the cycle (reviewed in Koob, 2013). The dysfunction caused by this addiction cycle shifts the motivation to drink based on positive feelings towards the avoidance of negative ones that occur during withdrawal, contributing even more to dependence states.

In humans, the first stages of alcohol intoxication have anxiolytic and euphoric effects, happening with BAC ranging from 5 to 12 mM (23 to 55 mg/dl). At BAC of 18 mM (80 mg/dl), which is reached after binge drinking, the effects shown are slowed reaction times, motor incoordination, and cognitive impairment. At BAC up to 50 mM (230 mg/dl), locomotor disruption, cognitive impairment, and sedation escalate. Above this level, individuals can show strong sedation and respiratory depression which can lead to coma or death. BAC higher than 100 mM (460 mg/dl) can be lethal, although chronic exposure to alcohol can increase tolerance and individuals can reach a BAC of 300 mM (or 1382 mg/dl) (Abrahao et al., 2017). When episodes of intoxication are intense and BAC reach 80 mg/dl, we have what is called binge drinking. Episodes of binge drinking usually happen after the consumption of 4-5 drinks within two hours (https://www.niaaa.nih.gov/alcohol-facts-and-statistics), and a drink contains the equivalent of 14 g of pure alcohol (https://www.niaaa.nih.gov/alcohol-health/overview-alcohol-consumption/what-standard-drink).

The initial alcohol intoxication, like other drugs of abuse, increases dopamine release in the nAcc. With repeated episodes of intoxication, dopamine can trigger neuroadaptation in the reward system and promote conditioned reinforcement, habit formation and increased compulsion to seek more alcohol (Koob & Volkow, 2016). The next stage is the withdrawal
period, in which the drug intake is suspended. The withdrawal period consists of negative emotional elements, such as chronic irritability, emotional pain, malaise (feeling of discomfort, illness, or uneasiness), dysphoria (state of unease or dissatisfaction), alexithymia (difficulty in identifying and describing feelings), states of stress, and loss of motivation for “natural” rewards, such as food (Koob & Volkow, 2016). During withdrawal from chronic drug exposure, neuroadaptation processes occur such as decreased dopaminergic and serotonergic transmission in the nAcc (Weiss, Lorang, Bloom, & Koob, 1993). In alcohol-dependent humans and rodents, there is a long-lasting decrease in dopamine D2 receptors in the nAcc (Koob, 2013). Such decreases in reward system function might contribute to the clinical syndrome of acute withdrawal and protracted abstinence and could also explain the loss of interest in normal, non-drug rewards (i.e., narrowing of the behavioural repertoire toward drugs and drug-related stimuli).

Finally, the preoccupation and anticipation stage is key in inducing relapse, in which the individual reinstates drug-seeking behaviour after abstinence. Relapse occurs due to behavioural inflexibility, poor executive control and strong association between drugs and cues that can induce drug reinstatement (Koob & Volkow, 2016). In rats, the PFC (mainly prelimbic cortex, and some infralimbic cortex) sends glutamatergic projections directly to mesocortical dopamine neurons in the VTA and nAcc, thus exerting excitatory control over dopamine cell firing and dopamine release in the PFC (reviewed in Geisler & Wise, 2008). Evidence from rodent studies suggests that increases in the PFC activity could elicit a strong glutamatergic response that mediates craving-like responses during the preoccupation/ anticipation stage (Koob & Volkow, 2016).
Factors that contribute to alcohol addiction

One of the main factors that contributes to alcohol intake is stress, and stress can influence any stage of the addiction cycle. Stress is generally defined as any stimulus that disrupts the body’s internal balance or homeostasis, and in reaction to a stressor experience a hormonal response is mounted in order to restore homeostasis (reviewed in McEwen & Gianaros, 2011). There are two main response systems that are triggered by stressors: the sympathetic nervous system, secreting epinephrine and norepinephrine, and the hypothalamus-pituitary-adrenal axis (HPA axis), secreting glucocorticoids, such as cortisol in humans and corticosterone in rats. Both systems are activated during acute stress (short duration), but the main system that is activated during chronic stress is the HPA axis, so more emphasis will be given to it in the next section. During chronic drug exposure, the HPA axis becomes dysregulated and elevated levels of corticosterone and corticotropin releasing hormone (CRH, the initial hormone in the HPA axis leading to the release of corticosterone from the adrenal) are found during withdrawal. Blockade of CRH receptors by antagonists reduces anxiety-like, stress-like effects of drug withdrawal and excessive drug taking during compulsive drug seeking in animals (reviewed in Koob et al., 2014).

HPA axis functioning

The activation of the HPA axis starts with inputs to the paraventricular nucleus (PVN) of the hypothalamus, that produces and secretes corticotropin releasing hormone (CRH) into the pituitaryhypophysyal portal system to the anterior pituitary. There, CRH binds to its receptors and activates the production and release of adrenocorticotropic hormone (ACTH) into the bloodstream. This hormone reaches the granular layer cortex of the adrenal glands, binds to its
receptors and activates the release of glucocorticoids into circulation (mainly cortisol in primates and corticosterone in rodents) (McEwen & Gianaros, 2011).

Glucocorticoids execute their actions via two types of cytosolic receptors: the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). Under physiological conditions, or under low concentrations of glucocorticoids, most MR are occupied by glucocorticoids whereas most GR remain available, given that MR have high affinity and GR have low affinity for the ligand. However, under stress conditions, glucocorticoid concentrations increase and mostly bind to GR, because MR are mostly occupied. Therefore, GR are considered the main receptors involved in stress responses (de Kloet, Vreugdenhil, Oitzl, & Joëls, 1998).

Glucocorticoids are steroid hormones and can cross the plasma membrane. When bound to GR, a complex is formed that translocates to the nucleus of the cells where GR can bind to specific regions on the DNA (GR responsive elements, GRE) and activate or inhibit the transcription of many different genes. The HPA axis activity is suppressed by a negative feedback loop, in which glucocorticoids bind to GR at each level of the axis and in extra-hypothalamic structures such as the hippocampus and the PFC, inhibiting the release of CRH and ACTH (reviewed in Herman, 2013).
Figure 1-3. Schematic representation of the HPA axis and the negative feedback loop. Arrows indicate the release of hormones, and bars indicate inhibitory action of glucocorticoids on their receptors. ACTH = adrenocorticotropic hormone, CRH = corticotropin releasing hormone, GC = glucocorticoid, GR = glucocorticoid receptor, Hipp = hippocampus, PFC = prefrontal cortex, PVN = paraventricular nucleus of the hypothalamus.
Glucocorticoid effects on brain and behaviour

Under acute stress, glucocorticoid concentration raises, reaches a peak, and then returns to baseline levels (or levels prior to the stress). The timeline of these events are dependent on the type of stressor (Lui et al., 2012). The increase in glucocorticoids can promote adaptive changes, such as increasing glucose levels in the blood, suppressing immune responses and other non-essential functions such as growth and reproduction. It can also induce changes in risk assessment, locomotion and aggressive behaviours that ultimately promote survival when confronted with the stressor (reviewed in Herman, 2013; Oitzl, Champagne, van der Veen, & de Kloet, 2010). However, under chronic or repeated stress, the concentrations of corticosterone remain higher for prolonged periods of time, which can have detrimental effects on many of the body’s systems, including brain structure and function, altered metabolism, suppressed immunity, cognitive deficits, and psychiatric disorders (reviewed in McEwen, Nasca, & Gray, 2016).

The exposure to stressors and the constant activation of the HPA axis can have an impact on the reward system and increase the propensity to drug-related problems (reviewed in Piazza & Le Moal, 1998; Sinha, 2001). For example, CRH antagonists prevent self-administration of many drugs of abuse in drug-dependent rats (Koob et al., 2014). Also, hypothalamic nuclei send innervations to limbic structures involved in reward processing, and the increase in glucocorticoid concentration due to a stressor can modulate the activity of dopaminergic neurons in the reward brain structures, affecting how the brain perceives rewards (Polter & Kauer, 2014). Most of the literature focus on adult animals, but there is increasing evidence that the developing adolescent brain might be particularly vulnerable to the effects of stressors on the reward system,
and be more susceptible than adults to drugs of abuse, especially alcohol (Burke & Miczek, 2014; Goldstein, Déry, Pilgrim, Ioan, & Becker, 2016).

**Adolescence**

Adolescence is the transitional developmental period between childhood and adulthood, which encompasses puberty but does not have clear markers of onset and end in both humans and rats (reviewed in Spear, 2000). Adolescence is period with a different behavioural repertoire than in earlier and later stages of the lifespan. Among these behaviours are increased novelty-seeking and risk-taking behaviours (Meaney & Stewart, 1981; Spear, 2000), which seems to have the advantage of allowing juvenile animals to explore beyond nest/parental area and leave to start a new group or join a different group than their parents, avoiding inbreeding (Spear, 2000). Also, adolescents shift from interactions with the parents to peers and rats experience more reward in peer interactions during adolescence than during adulthood (Douglas, Varlinskaya, & Spear, 2004; Trezza, Baarendse, & Vanderschuren, 2010). The exact span of adolescence varies across different species likely due to genetic, environmental and social factors, and mapping this developmental stage in rodents to the same stage in humans is challenging. Figure 1-2 illustrates this parallel.
In humans, the onset of puberty marks the transition from childhood to adolescence, which is defined by the World Health Organization as from about the age of 10 to 19 (https://www.who.int/maternal_child_adolescent/topics/adolescence/development/en/). During puberty, the activity of the hypothalamus-pituitary-gonadal axis increases and gonadotrophin releasing hormone (GnRH) is released from the hypothalamus. The rise in GnRH culminates in the production and release of steroid hormones from the gonads, mainly androgens in boys and estrogens in girls, which will confer secondary sexual characteristics to the developing body and have organizational effects on the brain and behaviour. Puberty can start in girls around 8-10 years of age and a bit later in boys (reviewed in McCormick, Green, & Simone, 2017).

In rats, the onset of puberty does not coincide with the onset of adolescence, and there is a prepubertal and postpubertal period. In the laboratory, rats are typically weaned at postnatal day (PND) 21, and physical markers of puberty are visible in females at about PND 35, and in males at about PND 42 (McCormick et al., 2017). Most research in rats has focussed on the prepubertal period, between PND 28-42 (Spear, 2000), but parallels between males and females
are hard to draw given the earlier onset of puberty in females. Therefore, some researchers have defined adolescence with an extended prepubertal and postpubertal timeframe, from PND 21 to 59. Adolescence can also be further divided into early (21-34), mid (34-46) and late period (46-60), and young adulthood is defined as PND 60 when sexual maturity is attained (Laviola, Macri, Morley-Fletcher, & Adriani, 2003; Tirelli, Laviola, & Adriani, 2003), although there is some ongoing brain maturation beyond that age.

Childhood (or neonatal period in rats) has been considered a critical period of development for decades, given the brain high plasticity during this period, and it is well-established that perturbations during this stage can lead to long-lasting consequences into adulthood. When the animal reaches adolescence, numerous brain systems are still maturing but in a different way. The excess of neurons and synapses are highly pruned, so only neuronal circuits that have been used can remain and be strengthened. Neurotransmitter systems are also maturing, and more receptors are produced. Among brain structures still undergoing maturation during adolescence are the hippocampus, amygdala, PFC and many limbic areas. As a consequence of the ongoing neuronal maturation, there is a change in cognitive function (Brenhouse & Andersen, 2011; Casey et al., 2010; McCormick & Mathews, 2010), which reflects the behavioural alterations characteristic of the adolescence period. Given the high plasticity of the developing adolescent brain, including the HPA axis and the reward system, this period is highly vulnerable to external events, such as stressors and drugs of abuse.

The HPA axis during adolescence

During adolescence, the maturation of the HPA axis results in a different functioning of this system. In response to acute stressors, adolescent rats typically show greater, or more
prolonged, release of ACTH and corticosterone than do adult rats, even though baseline levels are similar between ages (reviewed in Green & McCormick, 2016; Romeo, Patel, Pham, & So, 2016). However, not all stressors produce this age-specific effect. For example, 1 hour of social isolation increases corticosterone concentrations to the same extent in adolescent (PND 30) and adult (PND 70) rats (Hodges & McCormick, 2015). Restraint stress for 30 minutes produces prolonged release of corticosterone in prepubertal adolescent (PND 28) compared to adult rats (PND 77) (Romeo, Lee, Chhua, McPherson, & McEwen, 2004), whereas postpubertal adolescent rats (PND 45) have levels of corticosterone intermediate to prepubertal and adult rats (Green, Nottrodt, Simone, & McCormick, 2016; Green, Zeidan, Hodges, & McCormick, 2019). The secretion of ACTH also follows the same pattern as corticosterone for these age groups (Romeo et al., 2016). In response to chronic stressors, age also plays a role: when exposed to the same stressor (i.e., homotypic stressor) both adolescent and adult rats exhibit reduced corticosterone release (i.e., habituation), whereas when rats are exposed to homotypic stressor and then exposed to a novel stressor (i.e., heterotypic stressor), adolescent rats are more likely to show increased corticosterone release (i.e., sensitization) (Hodges & McCormick, 2015; Lui et al., 2012; McCormick, Merrick, Secen, & Helmreich, 2007).

Not only is the corticosterone secretion in response to stressors different, but also is the negative feedback system; although cytoplasmic levels of GR are similar between pre (PND 35), postpubertal (PND 45) and adult (PND 70) rats after restraint, there is greater translocation of GR to the nucleus in prepubertal rats compared to adults (Green et al., 2016). There are also changes in the maturation of the HPA axis, with adolescent rats (PND 28-30) having increased mRNA expression of arginine vasopressin (AVP) and CRH cell activation in the PVN (Romeo et al., 2006; Viau, Bingham, Davis, Lee, & Wong, 2005), and increased mRNA expression of
ACTH receptor in the adrenal glands in response to 30 min of restraint stress than adult rats (PND >60) (Romeo et al., 2014).

The reward system during adolescence

The neuronal changes that happen during adolescence mostly involve synaptic pruning, myelination, the overproduction and downregulation of receptors of many neurotransmitters, which result in modified brain circuits (reviewed in Brenhouse & Andersen, 2011). For example, dopamine innervation, receptors, synthesis and turnover peak during mid adolescence and are stabilized by the end of adolescence in the nAcc, striatum and PFC (reviewed in Burke & Miczek, 2014). Neurons containing GABA and GABA transporters steadily increase in the PFC after PND 15, reaching maximum levels in early adulthood (reviewed in Caballero & Tseng, 2016). Such changes in these neurotransmitter systems are happening in brain regions that are involved in reward and reinforcement (Koob & Volkow, 2016), which are critically implicated in susceptibility to the effects of drugs of abuse during adolescence.

During postnatal development, components of reward neurocircuitry are overproduced in early life followed by substantial elimination or pruning of this circuitry during adolescence. In rats, the amount of dopaminergic connections to the PFC increases throughout adolescence and reach final maturation around PND 60, while projections of the nAcc and striatum mature in early adolescence (Burke & Miczek, 2014). There is an increase in dopamine activity and turnover during early to mid-adolescence, with a clear peak in the PFC (PND 35) that declines by adulthood, whereas dopamine activity in nAcc and striatum remains high. Regarding dopamine receptors, there is a peak in D1 and D2 expression in the nAcc and striatum in early adolescence (PND 30) that is reduced by late adolescence, whereas peak expression in the PFC
occurs in late adolescence. Pruning and fine-tuning of dopaminergic systems in late adolescence is thought essential for proper development into adulthood (Andersen, 2003; Spear, 2000), as alterations to the pruning of DA systems may underlie the increased susceptibility to drug abuse (Crews, He, & Hodge, 2007; Sturman & Moghaddam, 2011).

As mentioned earlier, the maturation of brain systems is accompanied by behavioural changes in adolescence. The adolescence period is characterized by a reward-sensitive phenotype, whereby goal-directed behavior is dominant and rewards are particularly reinforcing. Such “reward-centricity” may not only favor a focus on primary rewards such as food, water, social, and, eventually, sexually attractive stimuli, but may direct behavior towards other rewarding stimuli as well (Doremus-Fitzwater & Spear, 2016). Yet, the reward-sensitive phenotype of adolescence may also impart a liability at this age, leading for example to patterns of binge drinking and escalated drug use. Such propensities for alcohol/drug use during adolescence may not only be encouraged by an adolescent reward-sensitive phenotype, but also by an attenuated sensitivity to aversive stimuli, including the aversive properties of alcohol, nicotine, and illicit drugs. For example, adolescent rats have higher basal dopamine levels in the nAcc and greater dopamine release in response to alcohol challenge than adults (Philpot & Kirstein, 2004; Philpot, Wecker, & Kirstein, 2009). In rats, exposure to alcohol during adolescence increases the propensity of addictive behaviour, but not during adulthood (McClory & Spear, 2014). This pattern of increased rewarding but decreased aversive sensitivities may help to promote high levels of alcohol/drug use among susceptible adolescents.
Adolescence and alcohol

Alcohol is one of the most consumed drugs in the world. According to the World Health Organization’s Global Health Observatory, 45 countries had a prevalence of alcohol use in 2016 higher than 60% of the population ([http://apps.who.int/gho/data/view.main.52480](http://apps.who.int/gho/data/view.main.52480)). From 2010 to 2016, alcohol use prevalence decreased in Australia (84% to 79.4%), Canada (77.1% to 64.5%), Germany (80.3 to 79.4%) and United Kingdom (83.9% to 73.4%), whereas it increased in the United States of America (68.9% to 71.7%) and Sweden (68.8% to 73%). Between 2015-2016, Canadians over 15 years of age consumed on average 8.2 litres of alcohol, and in some provinces the consumption was over 13 litres per year ([https://www.ccsa.ca/alcohol-canadian-drug-summary](https://www.ccsa.ca/alcohol-canadian-drug-summary)). Considering this same population in the same period, 24.4% of Canadians reported the heavy drinking of alcohol, which is the reporting of having consumed in one occasion five or more drinks in men or four or more drinks in women, at least once a month in the past year. Heavy drinking is more prevalent in Canadian men (65%) than in women (52%), and more prevalent in young adults (18-24 years old, 78%) than in adults (> 25 years, 56%).

Although the prevalence of alcohol use in the past 12 months among Canadian teenagers (12-17 years) has decreased from 45.4% in 2010-2011 to 39.5% in 2014-2015, the average onset age of drinking was 13.5 years ([https://www.ccsa.ca/alcohol-canadian-drug-summary](https://www.ccsa.ca/alcohol-canadian-drug-summary)). Early initiation of alcohol use is a predictor of alcohol use disorders later in life (Sartor et al., 2016), therefore the bases for these effects are worth investigating. About 30% of people under the age of 15 and 60% of people under the age of 18 have consumed alcohol at least once, and that more than 90% of teenagers’ alcohol consumption happens in the form of binge drinking (reaching blood alcohol levels greater than 80 mg/dl in two hours) ([https://www.niaaa.nih.gov/alcohol-health/overview-alcohol-consumption/alcohol-facts-and-statistics](https://www.niaaa.nih.gov/alcohol-health/overview-alcohol-consumption/alcohol-facts-and-statistics)). These data indicate a great
risk for teenagers to develop problems with alcohol addiction; the earlier the onset of drinking, the greater the risk of becoming dependent on alcohol (Bonomo, Bowes, Coffey, Carlin, & Patton, 2004).

This higher risk of alcohol dependence in the adolescent period in humans is also found in rodent models of drinking. Both adolescent humans and rats are less sensitive to the sedative effects of alcohol compared to adults, which is considered an important risk factor for alcohol use disorder (Crews, Vetreno, Broadwater, & Robinson, 2016). In keeping with evidence in humans (Spear & Swartzwelder, 2014), adolescent rats consume more ethanol per body weight than do adult rats (Doremus, Brunell, Rajendran, & Spear, 2005) and also experience more reward value from ethanol as well (Spear & Varlinskaya, 2010). Similar to evidence in humans, rats exposed to ethanol during adolescence show increased ethanol consumption in adulthood (e.g., Maldonado-Devincci, Alipour, Michael, & Kirstein, 2010). These parallels between humans and rats highlight the value of rodent models in investigating adolescence and alcohol use.

Further, ongoing brain development in adolescence may render adolescents more susceptible to negative consequences from exposures that would have little effect in adults. Thus, an important question is: what are the long-term consequences for ongoing brain development for alcohol exposures in adolescence? Therefore, the adolescent period brings new questions to bear, such as what are the factors that promote the consumption of alcohol in adolescence? Do the risk factors in adolescence differ from those in adulthood? Do the appetitive and consummatory responses to alcohol differ in adolescence than in adulthood? Do the immediate and long-lasting effects of alcohol differ at the two developmental points?
Animal models of adolescent alcohol exposure

Most animal models of adolescent ethanol exposure are conducted in rodents given the similarities in brain structures involved in reward and reinforcement (Burke & Miczek, 2014), the ability to use a large enough sample size, the convenience of obtaining rats at specific ages and the shorter lifespan, with adolescence to adulthood occurring within several weeks. There are many parallels between adolescence in rats and humans that make rats a good model for studies of alcohol. In both humans and rats, adolescents have a higher sensitivity to rewarding substances, such as sweets and drugs of abuse, than do adults (Doremus-Fitzwater, Varlinskaya, & Spear, 2010). In male rats, the peak of such sensitivity is approximately 50 days of age (Friemel, Spanagel, & Schneider, 2010). Human (Casey et al., 2010; Forbes & Dahl, 2010) and rat (Burton & Fletcher, 2012; Green & McCormick, 2013b) adolescents are more impulsive and have greater sensation seeking than adults. Further, the immaturity of the PFC, the neural region crucial for regulatory control and executive function, is thought to underlie the increased intake of alcohol in adolescence as well as increase the susceptibility of the PFC to the deleterious effects of alcohol exposure in both species (reviewed in Jadhav & Boutrel, 2019).

![Figure 1-5. Advantages and disadvantages of the most commonly used methods in the animal alcohol research literature. i.p. = intraperitoneal, s.c. = subcutaneous, i.v. = intravenous.](image-url)
The methods mostly used in animal alcohol research can be seen in Figure 1-3. The choice of the protocol used often depends on the research question. Many research questions are focused on factors that influence the propensity to consume alcohol as well as differences between adolescents and adults in the consumption of alcohol, and thus these rely primarily on voluntary consumption. Studies in which animals choose to ingest ethanol are more appropriate to assess motivational aspects of ethanol intake, since they produce changes in brain self-stimulating reward parameters that are not induced by experimenter-administered ethanol (Moolten & Kornetsky, 1990). Although rats will drink ethanol of varying concentrations in the absence of sweeteners, intake is greater when the ethanol is sweetened. The use of sweeteners such as sucrose and/or saccharin may better mimic the kinds of drinks consumed by teenagers (Johnston et al., 2018). In investigations of voluntary consumption, rats have access to a bottle containing water and a bottle containing ethanol of various concentrations, depending on the study, either continuously (2 bottle choice, 2BC) or intermittently (Intermittent Access to 2BC, IA2BC, in which ethanol is presented every other day). Extended periods of IA2BC are commonly used to model binge-drinking, with ethanol consumption increasing markedly over the weeks of exposure. Because consumption is voluntary and allows assessment of willingness to drink and motivational aspects of drinking, BAC higher than 80 mg/dl (that met the criterion for binge drinking) are not usually evident for several weeks. Most studies find that on a per body weight basis, adolescent rats consume more than adult rats (although see Wille-Bille, de Olmos, Marengo, Chiner, & Pautassi, 2017), irrespective of whether the ethanol is sweetened (Acevedo, Fabio, Fernández, & Pautassi, 2016). For example, early-adolescent rats (PND 28) provided with 10% ethanol solution in a 2BC model consumed more ethanol per body weight than did rats that started as adults (PND 71) (Vetter, Doremus-Fitzwater, & Spear, 2007). When
the percentage of ethanol increases from 6% to 20% as part of an IA2BC model, mid-adolescent male rats (PND 35) also consumed more than did adult male rats (PND 75), resulting in slightly higher BAC in the adolescents (Fernandez, Stewart, & Savage, 2016). Adolescent males also consumed more 15% ethanol than did adolescent females or adult rats in a 2BC model (Dhaher, McConnell, Rodd, McBride, & Bell, 2012). The greater consumption of adolescents than adults is also observed when ethanol is the only source of liquids in the cage (10% ethanol, (Bergstrom, McDonald, & Smith, 2006); 2.5% ethanol (Waters & McCormick, 2011). A sex difference in alcohol consumption during adolescence has been reported in humans as well, although the direction of the sex difference varies depending on age (Windle, 2016). Indeed, in the younger adolescent (8th grade), girls report drinking more than boys, whereas in the 12th grade the opposite was found. This sex difference may reflect the difference in sexual maturity between boys and girls, as girls mature first. Peers have a notable influence on alcohol consumption in teenagers, and the composition of the social networks (e.g., number of males versus females) influence the genders differently (reviewed in Jacobs, Goodson, Barry, & McLeroy, 2016). For example, a male teen’s drinking influences that of the female teen more so than vice versa (Deutsch, Steinley, & Slutske, 2014).

In rats, self-administration of ethanol in operant chambers allows for greater investigation of motivational aspects related to ethanol drinking than does intake in a voluntary consumption paradigm. By manipulating the ratio in which lever presses correspond to the administration of ethanol, researchers can determine the breaking point (or how many presses until the animal gives up) for each individual animal, the continuation of drug seeking when unavailability is signalled, and the effect of punishment on the continuation of ethanol use (Jadhav, Magistretti, Halfon, Augsburger, & Boutrel, 2017). Adolescent rats will make more lever presses, ingest
more ethanol and perform more goal-directed or habitual-seeking than adults (Serlin & Torregrossa, 2015).

Investigations of how exposure to ethanol, and the extent of exposure to ethanol, in adolescence influences consumption in adulthood has involved procedures other than voluntary consumption to manipulate adolescent exposure. Voluntary consumption in adolescence, however, is more likely to promote ethanol intake weeks later than is voluntary consumption in adulthood in both male (Siegmund, Vengeliene, Singer, & Spanagel, 2005) and female rats (Füllgrabe, Vengeliene, & Spanagel, 2007). In some studies, ethanol is provided as the only available liquid. For example, a 30-min access every other day of 10 or 20% ethanol sweetened with a “supersac” solution (3% sucrose, 0.125% saccharin) from PND 28-42 resulted in rats drinking more ethanol in adulthood with resulting BAC of 20-90 mg/dl, and with no change in the intake of sweetened solutions without ethanol (Broadwater, Varlinskaya, & Spear, 2013). This increased intake of ethanol in adulthood is also observed when late-adolescents (PND 51-58) are exposed to 8% ethanol for one week (Milivojevic & Covault, 2013). In addition to providing ethanol as the only available liquid, gavage, injection, or ethanol vapour are used to ensure a specific exposure within a determined time frame. High BACs that are comparable to binge-drinking concentrations can be achieved by using repeated intraperitoneal (i.p.) injections of ethanol. For example, adolescent rats (PND 25) injected 8 times with 3 g/kg of ethanol in a “2 days on 2 days off” model (which produces BAC of 130-207 mg/dl) later showed greater voluntary consumption of ethanol than did rats injected with saline (Pascual, Boix, Felipo, & Guerri, 2009). The administration route of injection, however, does not mimic the normal gastrointestinal absorption route in which ethanol is typically consumed. Therefore, the “binge” induction of high BACs can also be achieved by gavage. For example, adolescent rats (PND 28)
exposed 12 times with 5 g/kg of ethanol in a “4 days on, 3 days off” model consumed more ethanol as adults than did water-treated rats (Maldonado-Devincci et al., 2010). When the “binge” model is applied using injection or gavage to adolescent rats, the procedure has been referred to as adolescent intermittent ethanol (AIE) exposure, whereas the application to adult rats is referred to as chronic intermittent ethanol (CIE) exposure (Spear & Swartzwelder, 2014). Thirty minutes after one injection of 1 g/kg produced average BAC of 110 mg/dl in both adolescent and adult rats (Varlinskaya & Spear, 2002), whereas a 3 g/kg injection produced an average BAC of 195 in adolescents and 165 mg/dl in adults (Pascual et al., 2009). Using gavage, one dose of 2 g/kg produces BAC of 200 mg/dl 30 min after administration in adolescent rats, with slightly lower BAC in adults (around 150 mg/dl) (Pautassi, Myers, Spear, Molina, & Spear, 2008). Another consideration is that BACs do not necessarily reflect brain ethanol concentrations, which are slightly lower than found in blood (Pautassi et al., 2008).

**Factors that contribute to adolescent alcohol exposure**

The exposure to stressors also is a known risk factor for substance abuse (reviewed in Koob, 2014). In humans, acute stress exposure is associated with the use of alcohol as a coping mechanism, and although chronic stress exposure reduces the hedonic value of alcohol, chronic stress increases habitual responses to alcohol, which may drive alcohol compulsion and addiction (reviewed in Blaine & Sinha, 2017). In rodents, although stressors typically increase the self-administration of cocaine, heroin, morphine, nicotine and amphetamine (reviewed in Sinha, 2001), the effects for ethanol are inconsistent, and depend on the model and duration of stress, and whether or not the animal had been previously exposed to ethanol, and the testing age. For example, adult rats drinking ethanol after inescapable footshock increase intake compared to
non-stressed rats, but when ethanol consumption is already established, rats do not change their consumption after stress exposure (Meyer, Long, Fanselow, & Spigelman, 2013).

Most studies isolate the rats either temporarily or through single housing to gauge an individual’s ethanol consumption. The problem of using isolation is that it is a stressor, and more-so for adolescent rats than for adults (e.g., Hodges & McCormick, 2015). Thus the higher intake of adolescents than adults in some studies may reflect differential stress responding rather than specific age differences in willingness to consume, per se. Nevertheless, not all stressors increase ethanol intake. For example, daily footshock for 14 days (Brunell & Spear, 2005) and repeated social defeat stress (van Erp & Miczek, 2001) suppressed the consumption of sweetened alcohol in male adolescent rats. The suppression, however, may reflect a stress-induced reduction in the hedonic value of sweet substances (Konkle et al., 2003). In prepubertal adolescent rats, repeated restraint stress enhanced ethanol consumption in females and suppressed consumption in males, thus sex is also a factor in consideration (Wille-Bille, Ferreyra, et al., 2017). Stress, particularly early life stress, is a known risk factor for alcohol use disorders in humans (Enoch, 2011). In humans, although chronic stress reduces the hedonic value of alcohol, it can promote alcohol use by increasing habitual responses to alcohol (Blaine & Sinha, 2017). Although the presence of a peer may reduce the possibility of stress-induced ethanol consumption, there is evidence that the presence of peers may promote ethanol intake in rodents, and more so in adolescence than in adulthood and more so in male rats than in female rats (Logue, Chein, Gould, Holliday, & Steinberg, 2014; Varlinskaya, Truxell, & Spear, 2015a). In teenagers, the presence of a peer was found to increase the perception of the value of rewards (Smith, Steinberg, Strang, & Chein, 2015), and peers influence alcohol consumption in teenagers (Huang et al., 2014; Trucco, Colder, & Wieczorek, 2011). Thus, methodological factors such as
stressors and social context are important considerations in the interpretation of results in rodent models, and also highlight the parallels of evidence in rodents with evidence in humans.

**The effects of stress during adolescence on subsequent ethanol intake**

There are many different stressors used in the animal literature, which can be divided into physical stressors (restraint, footshock, cold) and psychological stressors (social defeat, social isolation, social instability), or can be a combination of different types (chronic variable/unpredictable stress) (McCormick et al., 2017). Social stressors are particularly relevant when studying adolescence, given that both humans and rodents are highly social species, and changes in the social environment caused by such stressors can alter the behavioural repertoire and have long-lasting consequences (reviewed in Burke, McCormick, Pellis, & Lukkes, 2017). For example, prolonged social isolation (PND 28-72) increases anxiety-like behaviour and ethanol consumption compared to group-housed rats in adulthood (Chappell, Carter, McCool, & Weiner, 2013). Social defeat from PND 35-39 can increase locomotion and reduce dopamine release into the nAcc induced by acute amphetamine in early adulthood (PND 56) (Burke, Forster, Novick, Roberts, & Watt, 2013). Thus, the adolescent brain may be particularly malleable by chronic stressful experiences and susceptible to their effects.

The stress model chosen for this thesis was the social instability stress (SS, reviewed in McCormick, 2010). This model consists of isolating pair-housed rats for 1 hour and then returning them to a different cage partner that is also undergoing the same procedure every day from PND 30 to 45. The rats are then returned to their original cage partner after the 16th isolation, and tests can be performed soon after to investigate immediate effects of SS or wait until rats reach adulthood to investigate long-lasting effects of SS. Figure 1-6 illustrates a scheme
of the overall SS procedure, as well as the schedule used to organize the new cage partners across the SS procedure.

**Figure 1-6.** The social instability stress (SS) procedure. Top: a schematic representation of the SS procedure, which involves a daily one hour isolation followed by change of cage partners from PND 30-45. Bottom: an illustration of the schedule to organize the new cage partners across the SS procedure. This schedule is for pairing 24 rats, and other sample sizes require different schedules.
This model is considered a mild stressor compared to other social stressors such as social defeat and isolation housing, and it has age-specific effects that appear only when SS is performed during adolescence. For example, when comparing rats that underwent SS procedure during adolescence (PND 30-45) or adulthood (PND 70-85), rats of both ages that had been returned to the same cage partner (ISO group) showed lower corticosterone secretion in comparison to control rats that were isolated for 1 hour once on either PND 45 or 85, which indicates a habituated response to a homotypic stressor (or reduced corticosterone release). However, adolescent rats that were returned to a different cage partner every day (SS group) showed sensitized corticosterone release after 1 hour with the new partner after isolation, whereas rats undergoing this procedure as adults showed a habituated response (Hodges & McCormick, 2015). This stress model also changes the behavioural repertoire of the rats: SS rats spend less time in social interactions when tested as adolescents (Hodges et al., 2017; Hodges, Baumbach, & McCormick, 2018) or as adults (Green, Barnes, & McCormick, 2013), but do not differ in social approach from control rats (both readily investigate rats that are separated by a mesh). SS rats show increased anxiety (McCormick, Smith, & Mathews, 2008) and impaired spatial long-term memory when tested as adults (but not at adolescence) (Green & McCormick, 2013a; McCormick, Nixon, Thomas, Lowie, & Dyck, 2010; McCormick et al., 2012). SS rats also show increased locomotor sensitization to psychostimulants (nicotine and amphetamine) both soon after the SS procedure in adolescence and several weeks later as adults relative to control rats (Mathews, Mills, & McCormick, 2008; McCormick & Ibrahim, 2007; McCormick, Robarts, Gleason, & Kelsey, 2004; McCormick, Robarts, Kopeikina, & Kelsey, 2005). SS rats also have an increased motivation for sweetened condensed milk and display more aggressive
behaviour when competing for it in adulthood (Cumming, Thompson, & McCormick, 2014). These results indicate a possible modulation of the reward system after rats are exposed to SS. We do not know, however, whether these SS effects would also affect the consumption of another drug of abuse, alcohol.

As mentioned before, the effects of stress on the consumption of ethanol are mixed and the same mixed effects are found in adolescent stress studies. Adolescent social isolation usually increases ethanol intake both at adolescence and adulthood (Butler, Carter, & Weiner, 2014; Ehlers, Walker, Pian, Roth, & Slawecki, 2007; Juárez & Vázquez-Cortés, 2003; Lesscher et al., 2015; McCool & Chappell, 2009; Schenk, Gorman, & Amit, 1990; Skelly, Chappell, Carter, & Weiner, 2015; Van Waes et al., 2011), but some studies have found no difference or lower ethanol consumption between isolated and group-housed rats (Doremus et al., 2005; Lodge & Lawrence, 2003; Thorsell, Slawecki, Khoury, Mathe, & Ehlers, 2005). Stress by footshocks decreases ethanol intake in adolescent rats (Brunell & Spear, 2005), restraint stress during adolescence decreases (Wille-Bille, Ferreyra, et al., 2017) or does not change (Acevedo et al., 2016) ethanol intake during late adolescence, and social defeat during late adolescence increases ethanol intake in adulthood (Riga et al., 2014). Therefore, the method of stress used can have opposing effects regarding ethanol intake and studies must be compared with caution.

In the present work, voluntary alcohol drinking methods were used in order to assess the effects of adolescent social instability stress. We wanted to minimize the stress involved with invasive methods such as injection and gavage, and to be able to evaluate the motivational aspects that could not be evaluated with administration techniques such as vapor inhalation. We opted to not use operant conditioning to assess the motivation for ethanol given the prolonged period of training rats involved limited the ability to assess intake during adolescence.
Goals of the thesis

The main goal of this thesis was to investigate the effects of adolescent social instability stress (SS) on immediate and long-lasting reward-related behaviours in male rats using voluntary alcohol intake paradigms. My hypothesis was that social instability stress would modify the reward system and affect the propensity to drink alcohol soon and long after the stress was concluded. The propensity to drink would also be affected by the social environment in which the drinking was taking place (alone or with a peer). As a comparison, I also investigated the intake of another rewarding substance, sucrose, with the prediction that if social instability stress alters the development of the reward system, then differences between SS and control rats would be evident for sucrose intake as well.
Chapter 2: Adolescent social stress and social context influence the intake of ethanol and sucrose in male rats soon and long-after the stress exposures.

This chapter has been adapted from the published article:


Author contribution: for this manuscript, Dr Cheryl McCormick and I designed the experimental and formulated the research questions. I wrote the proposal for the ethics approval, ordered the rats required for the experiment, performed the social instability stress procedure on rats (with help from Travis Hodges), performed the behavioural experiments (with help from Travis Hodges and Jennet Baumbach), scored videos, analyzed the data, performed the statistics, I helped to create the figures, wrote the paper and edited after receiving feedback from Dr Cheryl McCormick.
1. Introduction

The hypothalamus-pituitary-adrenal (HPA) axis is one of the main stress-responsive systems of the body, which, when activated, results in the elevation of glucocorticoid hormones (mainly corticosterone in rats) into circulation (reviewed in McEwen & Gianaros, 2011). In response to an acute stressor, corticosterone is released from the adrenal cortex, binds to glucocorticoid receptors (GR) in the periphery and in the brain, and promotes adaptive changes. Examples of such adaptive changes include increasing glucose levels in the blood, suppressing immune responses and other non-essential functions such as growth and reproduction, as well as changes in risk assessment, locomotion and aggressive behaviours (reviewed in Herman, 2013; Oitzl et al., 2010). Corticosterone release is terminated via negative feedback actions at GR receptors at all levels of the HPA axis as well as at extra-hypothalamic sites, notably the hippocampus and the prefrontal cortex (reviewed in Herman, 2013). Under chronic or repeated stress, the concentrations of corticosterone remain higher for prolonged periods of time, which can have detrimental effects on many of the body’s systems including brain structure and function (reviewed in McEwen et al., 2016). These effects are typically greater during times of relatively greater brain development, such as perinatal life (reviewed in McEwen & Morrison, 2013). Adolescence also is a time of greater brain development relative to adulthood, and increasingly is recognized to be an additional time of greater sensitivity to stressors and glucocorticoids than in adulthood (reviewed in McCormick, Hodges, & Simone, 2015). In addition there is greater, or more prolonged, release of glucocorticoids to a variety of stressors in adolescence than in adulthood (reviewed in Romeo et al., 2016; Spear, 2000). Thus, the adolescent brain may be particularly malleable by stressful experiences.
Adolescence is a time of development of the behavioural repertoire, and many behavioural differences between adolescents and adults have been characterized. For example, adolescent rats display greater risk-taking and novelty-seeking behaviour and social play than do adult rats (reviewed in Spear, 2000). In humans, adolescents show greater orientation away from parents and towards peers and increased risk-taking activities compared to adults (reviewed in Schriber & Guyer, 2016; Shulman et al., 2016). During adolescence, rats spend more time in social interaction than in adulthood (Meaney & Stewart, 1981), and adolescents experience greater reward value from social interaction than do adult rats (Douglas et al., 2004). Adolescents are also more sensitive to natural rewards and to drugs of abuse than are adults (reviewed in Spear & Varlinskaya, 2010). Moreover, adolescents are less sensitive to negative effects of ethanol intake compared to adults, which could be one mechanism for their increased intake relative to adults (reviewed in Spear & Varlinskaya, 2010).

Ethanol is consumed to a greater extent in adolescent than in adult rats across a variety of experimental conditions (Doremus et al., 2005). Additionally, adolescent rats have a higher consumption of sweet substances than do adults, with a peak in consumption at postnatal day 50 (late adolescence) (Friemel et al., 2010). Adolescent rats also show greater positive responses to sucrose solutions than do adults using measures of taste reactivity (Wilmouth & Spear, 2009). The heightened sensitivity to rewards in adolescents may be a risk factor for drugs of abuse; for instance, the earlier the onset of drinking, the greater the risk of becoming dependent on ethanol (reviewed in Crews et al., 2016). In adult humans, consumption of alcohol can happen as a coping mechanism (Dvorak, Pearson, & Day, 2014), whereas in young adults the main reason to drink alcohol seems to be because of social motives, such as peer pressure (Kuntsche, Knibbe, Gmel, & Engels, 2005). In humans, the presence of peers increases the perception of the value of
rewards in adolescents: In a low risk-taking situation, adolescents watched by peers had increased activation of the ventral striatum, a region involved in the reward system, compared to adolescents who were alone and compared to adults watched by peers (Smith et al., 2015). In rodents, the presence of peers increased the intake of ethanol compared to rats that were drinking alone (Logue et al., 2014; Varlinskaya et al., 2015a). Thus, adolescence and the social context influence the risk of substance abuse.

The exposure to stressors also is a known risk factor for substance abuse (reviewed in Koob, 2014). In humans, acute stress exposure is associated with the use of alcohol as a coping mechanism, and although chronic stress exposure reduces the hedonic value of alcohol, chronic stress increases habitual responses to alcohol, which may drive alcohol compulsion and addiction (reviewed in Blaine & Sinha, 2017). In rodents, although stressors typically increase the self-administration of cocaine, heroin, morphine, nicotine and amphetamine (reviewed in Sinha, 2001), the effects for ethanol are inconsistent, and depend on the model and duration of stress, and whether or not the animal had been previously exposed to ethanol, and the testing age. For example, adult rats drinking ethanol after inescapable footshock increase its intake compared to non-stressed rats, but when ethanol consumption is already established, rats do not change their consumption after stress exposure (Meyer et al., 2013).

Most research of the effects of stress on alcohol intake in preclinical models has involved adult animals or prepubertal adolescents (< 40 days of age) (Brunell & Spear, 2005; Meyer et al., 2013; Siegmund et al., 2005; Wille-Bille, Ferreyra, et al., 2017). It is important to investigate later ages of adolescence in preclinical studies because adolescence in humans is defined as beginning at puberty (World Health Organization, [link](https://www.who.int/maternal_child_adolescent/topics/adolescence/development/en/)). The
available research in adolescence mostly has involved social isolation housing (Butler, Carter, et al., 2014; Doremus et al., 2005; Schenk et al., 1990; Skelly et al., 2015; Van Waes et al., 2011). Although often referred to as a social stressor, social isolation housing does not involve the repeated, rapid, and prolonged elevation of corticosterone that usually identifies an event as a stressor, and may be better characterized as a severe form of “social malnourishment” (reviewed in McCormick et al., 2017, 2015; Montagu, 1977). Milder forms of social stress that elicit a rapid rise in corticosterone may have broader relevance for normative teenage development and risk.

Our model of social instability stress (SS) consists of daily one hour of isolation after which rats are housed with a new cage partner beginning at postnatal day (PND) 30 for 16 days, encompassing a prepubertal and postpubertal period of stress exposure that allows for the investigation of its effects soon after the termination of stress procedures while the rats are still adolescents. Whereas rats that are administered the SS procedure in adulthood rather than in adolescence habituate (i.e., reduce corticosterone release) to both the daily isolation and to the daily change of cage partner, adolescents have higher plasma corticosterone concentrations one hour after the 16th pairing with a new cage partner relative to age-matched rats undergoing their first isolation and return to a new cage partner (Hodges & McCormick, 2015); the increased plasma corticosterone is not attributable to aggressive exchanges, as there is little to no aggression observed in either controls or in SS rats in the homecage during the days of the procedure (Hodges & McCormick, 2015; McCormick et al., 2007). There is some evidence of altered behavioural responses to drugs of abuse in rats that undergo the SS procedure in adolescence, for example, heightened locomotor sensitization to amphetamine when tested in either adolescence or in adulthood (reviewed in McCormick, 2010). These studies, however, were limited by the fact that the drugs were administered to the rats, and thus do not address how
readily SS rats would self-administer the drugs. This limitation can be addressed using ethanol, which rats voluntarily consume. A study from Butler’s group (Roeckner, Bowling, & Butler, 2017) used our model of social instability stress to investigate its effects on intake of 20% ethanol (two-bottle choice paradigm) in male and female rats during late adolescence until adulthood. They found that SS male rats had higher preference for ethanol compared to other stressed groups (rats that returned to same cage partner or rats that were just isolated), whereas SS female rats did not differ ethanol preference from other groups. However, this study did not investigate the role of social context in the effects of SS on intake.

In the present study, we predicted that SS rats would consume more ethanol than would CTL rats. Further, based on the atypical social repertoire of SS rats (e.g., SS rats spend less time in social interaction than do CTL rats (Hodges & McCormick, 2018) and evidence that adolescent rats consume more ethanol when in the presence of peers than when alone (Varlinskaya et al., 2015a), we predicted that social context may be an important moderator in the differences between SS and CTL rats. We predicted greater differences between SS and CTL rats when in the presence of a peer than when alone. Rats were tested in three phases, each involving different social contexts: (I) access to 10% ethanol in a test apparatus either alone or in the presence of an unfamiliar peer (separated by mesh), (II) access to both water and 10% ethanol in a test apparatus in the presence of the cage partner (separated by mesh), and (III) in competition against the cage partner for access to 10% ethanol. We used unsweetened ethanol to avoid any effect of the sweetener. Stressors can also increase the intake of sweet substances (Adam & Epel, 2007), and we previously reported that when tested in adulthood after SS in adolescence, SS rats had a higher intake of sweetened condensed milk than did CTL rats, and were more aggressive with their cage partner in competing for access to the feeder than were
CTL rats (Cumming et al., 2014). To address whether the effects of SS are specific to ethanol or would also be evident with a sweet reward, in a separate experiment with different rats we also investigated consumption of 1% sucrose in the same three social context phases.

2. Materials and Methods

2.1. Social Instability Stress

Male Long Evans rats (n = 136 in 4 cohorts) were purchased from Charles River (USA) and arrived on postnatal day (PND) 23. To minimize the effects of shipping, our rats were shipped at the time of weaning when they would be undergoing the stress of change in housing that occurs at weaning. For these initial studies, we focused on male rats only given the large sample size required for the experiments. Nevertheless, given that the effects of SS are sex-specific, it will be important to also test females (McCormick, 2010). Rats were randomly pair housed in a 30 cm x 33 cm x 18 cm home cage with free access to food and water. The colony room was on a 12:12 hr light cycle (lights on at 05:00 am). Rats were left undisturbed until PND 30, when half of the rats underwent the social instability stress (SS) procedure, while the other half, control (CTL) rats, were left undisturbed except for regular cage maintenance (reviewed in McCormick, 2010). For the SS procedure, rats were isolated daily in a ventilated container (12 cm x 10 cm) for one hour from PND 30-45 and, after the isolation, rats were returned to a new cage partner every day, never meeting the same rat twice. After the 16th isolation, rats were returned to their original cage partner. The SS procedure was performed during the light cycle at a different time on each day to minimize habituation to the procedure. After PND 45, rats were randomly assigned either the 10% ethanol (EtOH) experiment (Experiment 1) or to the 1% sucrose experiment (Experiment 2), with cage partners assigned to the same experiment. All
procedures were approved by the Brock University Institutional Animal Care Committee (ACC) and were carried out in adherence to the Canadian Council on Animal Care guidelines.

Figure 2.1 illustrates the design and procedures of the experiments and the allocation of rats to experiments. Both experiments involved the same three phases.

**Figure 2-1.** Experimental groups and timeline of experimental procedures.

### 2.2. Phase I: Social context and intake of 10% EtOH (Experiment 1) or 1% sucrose (Experiment 2)

On PND 46, after 8 hours of water restriction and one hour after the onset of the dark cycle, 136 CTL and SS rats in both the EtOH experiment or the sucrose experiment were assigned to either the Alone condition or the Social condition. In each experiment, rats were placed individually in one side of a test arena (60 cm x 30 cm x 53 cm) divided in half by a mesh
insert (30 cm x 53 cm) in a room illuminated by a dim red light. The other side of the test arena was either empty (Alone condition) or contained an unfamiliar age- and group-matched peer (Social condition). The opposite wall of the test arena had an opening through which a spout could be extended from a bottle placed outside the cage. The bottle contained either a 10% EtOH solution (Experiment 1) or a 1% sucrose solution (Experiment 2). The session was 30 min long and was recorded by a camera mounted to the ceiling. Videos were assessed by an experimenter blinded to the experimental groups. Time spent licking the spout was used as the measure of intake based on previous research indicating a high correlation between time at spout and the volume consumed (Varlinskaya et al., 2015a). Time at the mesh dividing the apparatus in half was used to test whether rats were aware of the context (Social vs Alone), and it was used as the index of awareness of the peer.

72 of the 136 rats were tested again three weeks later at PND 70 (adults), to investigate whether any effects of adolescent social stress on EtOH and sucrose intake would be evident in adulthood after a lengthy “stress-free” period. Rats tested again on PND 70 were left undisturbed after the test session on PND 46 and were assigned to the same conditions and solutions as in their first test in adolescence.

2.3. Phase II: Choice of water and 10% EtOH (Experiment 1) or 1% sucrose (Experiment 2) when with the Familiar Cage Partner

This phase differed from Phase I in that all rats were tested in a social condition, although rather than the context involving an unfamiliar peer, it involved the familiar cage partner. In addition, a water alternative was provided alongside the EtOH (Experiment 1) or sucrose (Experiment 2) solution whereas in Phase I, only EtOH or sucrose was provided.
After Phase I, on PND 47 for the adolescent group or on PND 71 for the adult group, rats began Phase II. For 5 consecutive days, after 8 hours of water restriction and one hour after the onset of the dark cycle, cage partners were placed in the test arena compartments separated by mesh (same arena as in Phase I). In each one hour test session, rats in both compartments had access to two bottles: in Experiment 1, one bottle contained tap water and the other bottle contained 10% EtOH; in Experiment 2, one bottle contained tap water and the other one contained 1% sucrose. The room was illuminated by a dim red light and the session was recorded by a camera mounted to the ceiling. Videos were assessed by an experimenter blinded to the experimental groups. Latency to first drink and time spent licking each spout was measured as in Phase I.

2.4. Phase III: Drinking Competition for 10% EtOH (Experiment 1) or 1% sucrose (Experiment 2)

After Phase II, on PND 52 for the adolescent group or on PND 76 for the adult group, rats began the Drinking Competition task (Cumming et al., 2014; Malatynska, Pinhasov, Crooke, Smith-Swintosky, & Brenneman, 2007). The task consisted of one habituation session and five test sessions. All sessions began one hour after the onset of the dark cycle under dim red light. The apparatus consisted of two equally sized 20 cm x 16 cm x 16 cm chambers connected by a 40 cm x 10 cm x 10 cm hallway. At the centre of the hallway was a self-refilling feeder filled with either 10% EtOH (Experiment 1) or 1% sucrose solution (Experiment 2). The feeder had a 1 cm opening that allowed drinking access to only one rat at a time. In the habituation session, rats were habituated individually to the apparatus until they drank from the feeder (a maximum of 15 min). For test sessions, the feeder chamber was separated by two walls allowing each rat to be confined to an end of the apparatus away from the feeder. The walls were removed at the start of
a testing session at the same time to allow each rat the same access to the feeder. On 5 consecutive days, rats were placed in one chamber of the apparatus with their cage partner in the opposite chamber and, after the walls were removed, both rats could compete for access to the feeder for 5 min. The test sessions were recorded by a camera mounted to the ceiling and videos were evaluated by an experimenter blinded to the experimental groups. Based on our previous investigation with sweetened condensed milk (Cumming et al., 2014), we quantified the number of face whacks as the measure of aggression. A face whack was defined as one of the rats using its forepaw forcefully to strike the facial region of the other rat (Cumming et al., 2014). We also counted the number of times rats terminated the drinking bout voluntarily (in the absence of intervention by the other rat) or involuntarily (forced by the other rat either after a number of face whacks or from being shoved away by the partner’s body). This competition test was designed to evaluate dominant-submissive relationships (DSR) between pairs of rats (Berdo, Smith, & Macdonald, 1995). To determine whether a pair had a dominant-submissive relationship (DSR), paired t-tests were calculated on the time spent drinking of the cage mates across the five days. If the p-value was lower than 0.05 (one-tailed), the pair was considered to have a DSR (the rat that spent more time at the feeder was considered dominant, the other submissive, as in (Cumming et al., 2014). In the EtOH group, only 5 in 34 pairs met the criteria for DSR, whereas in the Sucrose group 15 out of 34 pairs met the criteria. Therefore, we did not include this measure in our statistical analyses. Nevertheless, our previous research indicated that although rats in a DSR displayed less aggression and greater consumption than did rats in a non-DSR, the effects of SS in competition for sweetened condensed milk were irrespective of the DSR status of the pair (i.e., main effect of DSR status, main effect of SS, no interaction) (Cumming et al., 2014).
2.5. Statistical analyses

Statistical analyses were performed using SPSS version 24 software and consisted of analyses of variance (ANOVA) or repeated measures ANOVA, where appropriate. For Phase I, between-subject factors were Stress (CTL, SS) and Social condition (Alone, Social) and the within-subject factor was Time point (10, 20, 30 min). Because only a subset of rats in Phase I was retested in adulthood, the age groups were analyzed separately. For Phase II, between-subject factors were Stress (CTL, SS) and Age (Adolescent, Adult), and the within-subject factor was Test day (1st day, 5th day). For Phase III, all measures were combined for a pair of competing rats, and between-subject factors were Stress (CTL, SS) and Age (Adolescent, Adult), and the within-subject factor was Test day (1st day, 5th day). Alpha was set at \( p < 0.05 \). Post-hoc analyses consisted of paired-sample or independent-sample t-test where appropriate.

3. Results

3.1. Phase I, Experiment 1: 10% EtOH intake and social context (alone or unfamiliar peer)

Adolescence: Adolescent SS rats had shorter latencies to drink (F\(_{1,63} = 6.31, p = 0.015\)) and drank more EtOH (F\(_{1,63} = 4.90, p = 0.030\)) than did CTL rats. Alone rats had shorter latencies to drink than did Social rats (F\(_{1,63} = 5.40, p = 0.023\)), but did not differ in intake (\( p = 0.108 \)). The two-way and three-way interactions of Stress group, Social context, and or Time point were not significant for either latency or intake (all \( p > 0.10 \)). See Figure 2-2(a) and (c).

Rats were aware of the social context in adolescence irrespective of Stress group: Alone rats spent less time at the mesh than did rats in the Social condition (F\(_{1,63} = 118.61, p < 0.0001\) and F\(_{1,63} = 88.57, p < 0.0001\)). Rats decreased time at the mesh across time points (F\(_{2,126} = 5.81, p < 0.005\) and F\(_{2,126} = 3.09, p = 0.049\)).
= 0.004), with all 3 time points significantly different from each other (all \( p < 0.001 \)). Other main effects and interactions were \( p > 0.179 \). See Figure 2-2(e).

**Adulthood:** SS rats had longer latencies to drink EtOH (\( F_{1,32} = 4.42, p = 0.044 \)), but did not differ in intake compared with CTL rats (\( p = 0.52 \)). Rats decreased the time at the spout over time (\( F_{2,64} = 14.46, p < 0.0001 \)), with all 3 time points significantly different from each other (all \( p < 0.009 \)). The main effect of Social condition and interactions were \( p > 0.107 \) for both latency and intake. See Figure 2-2(b) and (d).

Rats were aware of the social context in adulthood irrespective of stress condition in adolescence: Alone rats spent less time at the mesh than did Social rats (\( F_{1,32} = 21.42, p < 0.0001 \)). Other main effects and interactions were \( p > 0.19 \), except for Stress (\( p = 0.066 \)). See Figure 2-2(f).

### 3.2. Phase I, Experiment 2: 1% sucrose intake and social context (alone or unfamiliar peer).

**Adolescence:** SS and CTL rats did not differ in the latency to drink (\( p = 0.243 \)) or in the intake of 1% sucrose (\( p = 0.469 \)), and rats drank more when alone than when in the presence of a peer (\( F_{1,65} = 5.68, p = 0.020 \)). Intake changed over time (\( F_{2,130} = 6.10, p = 0.004 \)), whereby rats had an increase from 10 to 20 min (\( p = 0.003 \)), then a decrease from 20 to 30 min (\( p = 0.001 \)). Other main effects and interactions were \( p > 0.101 \), except for the three-way interaction of Time, Stress group, and Social condition on time at spout (\( p = 0.058 \)). See Figure 2-3(a) and (c).

Adolescent rats were aware of the social context irrespective of Stress group: Alone rats spent less time at the mesh than did Social rats (\( F_{1,65} = 93.02, p < 0.0001 \)). Rats decreased time at the mesh across time (\( F_{2,130} = 6.94, p = 0.001 \)), whereby rats spent less time from 10 to 30 min (\( p \))
< 0.001), but did not differ from 10 to 20 min (p = 0.051) or 20 to 30 min (p = 0.107). See Figure 2-3(e).

**Adulthood:** SS rats and CTL rats did not differ in the latency to drink (p = 0.381) or in the intake of 1% sucrose (p = 0.629), and there was no effect of Social condition, or interactions of Social condition, Stress, and Time point (all p > 0.10). Rats decreased the time at the spout across the session (F<sub>2,64</sub> = 31.71, p < 0.0001 and F<sub>2,64</sub> = 51.68, p < 0.0001), with all 3 time points significantly different from each other (all p < 0.001). See Figure 2-3(b) and (d).

Adult rats were aware of the social context irrespective of Stress group: Alone rats spent less time at the mesh than did Social rats (F<sub>1,32</sub> = 22.463, p < 0.0001). There was an effect of Time point on time spent at the mesh across time (F<sub>2,64</sub> = 7.01, p = 0.002), whereby rats decreased time from 10 to 20 min (p = 0.002) and increased time from 20 to 30 min (p = 0.006), with no difference between 10 and 30 min (p = 0.719). Other main effects and interactions were p > 0.22, except for the interaction of Time and Stress (p = 0.083). See Figure 2-3(f).

### 3.3. Phase II, Experiment 1: Choice of 10% EtOH and water when with the cage partner

Irrespective of age at time of testing, SS rats drank more EtOH than did CTL rats (F<sub>1,64</sub> = 8.86, p = 0.004), and did not differ in latency to drink (p = 0.45). Rats were slower to drink on the 5th day than on the 1st day (F<sub>1,64</sub> = 7.55, p = 0.008). Other main effects and interactions were p > 0.11, except for the main effect of Age (p = 0.092) and the interaction of Age and Test day (p = 0.072) on latency to drink, and the interaction of Stress and Age (p = 0.096) for time spent drinking. See Figure 2-4(a) and (c).

SS and CTL rats did not differ in intake (time spent drinking) of the water bottle (p = 0.963) or in latency to drink (p = 0.813). Rats were faster to start drinking on the 5th day
compared to the 1st day (F$_{1,64} = 5.48$, $p = 0.022$). There was an interaction between Test day and Age for time spent drinking water (F$_{1,64} = 15.16$, $p < 0.001$). Post-hoc analyses indicated that Adolescents and Adults did not differ in time spent drinking water on the 1st day ($p = 0.48$), and Adolescents increased their time spent drinking water from the 1st day to the 5th day (both $p < 0.001$) whereas Adults did not ($p = 0.07$), and Adolescents spent more time drinking water than did Adults on the 5th day ($p < 0.001$). See Figure 2-4(b) and (d).

3.4. Phase II, Experiment 2: Choice of 1% sucrose and water when with the cage partner

The main result was that irrespective of age at time of testing, SS and CTL rats did not differ in latency to drink ($p = 0.781$) or in intake ($p = 0.626$) of Sucrose.

Rats had shorter latencies to drink sucrose on the 5th day than on the 1st day (F$_{1,64} = 17.21$, $p < 0.001$). Other main effects and interactions were $p > 0.18$, except for the interaction of Age and Test day ($p = 0.058$). For the water bottle, rats had longer latencies on the 5th day than on the 1st day (F$_{1,64} = 4.06$, $p = 0.048$) and Adult rats had shorter latencies than did Adolescent rats. There was an interaction of Age and Stress group on latency to drink water (F$_{1,64} = 6.02$, $p = 0.017$). Post-hoc analyses indicated that adult CTL rats were faster than Adolescent CTL rats ($p = 0.002$), and Adolescent SS rats did not differ from Adult SS rats ($p = 0.448$). Adolescent CTL rats did not differ from Adolescent SS rats ($p = 0.117$), and Adult CTL were faster than Adult SS rats ($p = 0.020$). See Figure 2-5(a) and (b).

There was an interaction of Age and Test day for time spent drinking sucrose (F$_{1,64} = 20.78$, $p < 0.001$), which obviated the main effects of Test day (both $p < 0.001$) and Age ($p \leq 0.001$). Post-hoc analyses indicated that Adolescents and Adults did not differ in time spent drinking sucrose ($p = 0.12$) on the 1st day. Adolescents spent more time drinking sucrose ($p <$
than did Adults on the 5th day. Both Adolescents and Adults increased time spent drinking sucrose \((p < 0.001 \text{ and } p = 0.014)\) from the 1st to the 5th day. Other main effects and interactions were \(p > 0.15\), except for the interaction of Test day and Stress group \((p = 0.073)\). See Figure 2-5(c).

For the water bottle, there was only an effect of Test day, with rats drinking less water \((F_{1,64} = 15.343, p < 0.001)\) on the 5th than on the 1st day. Other main effects and interactions were \(p > 0.34\). See Figure 2-5(d).

### 3.5. Phase III, Experiment 1: Competition against the cage partner for 10% EtOH

The main results were that in adolescence, but not in adulthood, SS rats drank more EtOH and were more resistant to leaving the feeder (more involuntary retreats) than CTL rats.

The time spent drinking increased from 1st day to 5th day \((F_{1,30} = 14.81, p = 0.001)\), and Test day did not interact with other factors \((all \ p > 0.14)\). There was no main effect of Age \((p = 0.72)\), and the effect of Stress Group \((p = 0.023)\) was obviated by an interaction with Age \((p = 0.029)\). Post-hoc analyses indicated that among Adolescents, SS rats drank more than did CTL rats \((p = 0.003)\), and SS and CTL did not differ among Adult rats \((p = 0.13)\). There were no age differences in either CTL rats \((p = 0.39)\) or SS rats \((p = 0.19)\). See Figure 2-6(a).

Face whacks increased from 1st day to 5th day \((F_{1,30} = 47.66, p < 0.0001)\) and Adolescents did more face whacks than did Adults \((F_{1,30} = 5.06, p = 0.032)\). There was no effect of Stress \((p = 0.535)\) and all interactions were non-significant \((p > 0.12)\). Involuntary retreats increased from 1st day to 5th day \((F_{1,30} = 5.22, p = 0.030)\). No other main effect of interaction was significant \((p > 0.10)\) except for the interaction of Stress and Age \((F_{1,30} = 5.19, p = 0.030)\). Post-hoc analyses indicated that among Adolescents, SS rats made more involuntary retreats than did CTL rats \((p =
0.045), and the two groups did not differ among adults \((p = 0.48)\). The Age groups did not differ for either CTL \((p = 0.32)\) or for SS \((p = 0.06)\) rats. There were no group differences in the number of voluntary retreats. Main effects and interaction were \(p > 0.17\), except for Stress \((p = 0.068)\), Test day \((p = 0.073)\) and the interaction between Stress and Age \((p = 0.096)\). See Figure 2-6 (b), (c) and (d).

### 3.6. Phase III, Experiment 2: Competition against the cage partner for 1% sucrose

Irrespective of age, SS rats drank more sucrose than did CTL rats \((p = 0.002)\), and Adolescents drank more than did Adults \((p = 0.018)\). Time spent drinking increased from 1st day to 5th day \((F_{1,30} = 45.34, p < 0.0001)\), and Test day did not interact with other factors (all \(p > 0.20\)). Any interaction of these factors was not significant (all \(p > 0.78\)). See Figure 2-7(a).

Face whacks increased from 1st day to 5th day \((F_{1,30} = 23.82, p < 0.001)\) and Adolescents did more face whacks than did Adults \((F_{1,30} = 5.62, p = 0.024)\). There was no effect of Stress \((p = 0.839)\) and all interactions were non-significant \((p > 0.11)\), except the interaction of Stress and Age \((p = 0.084)\). There was no effect of Stress Group on either involuntary retreats \((p = 0.186)\) or voluntary retreats \((p = 0.38)\). The effect of Test day \((F_{1,30} = 56.45, p < 0.0001)\) and of Age \((F_{1,30} = 7.48, p = 0.010)\) on involuntary retreats was obviated by their interaction \((F_{1,30} = 5.56, p = 0.025)\). *Post-hoc* analyses indicated that Adolescents and Adults did not differ on 1st day \((p = 0.50)\), and Adolescents made more involuntary retreats than did Adults on 5th day \((p = 0.008)\). Involuntary retreats increased from 1st day to 5th day for both Adolescents \((p < 0.001)\) and Adults \((p = 0.001)\). Voluntary retreats increased from 1st day to 5th day \((F_{1,30} = 30.24, p < 0.0001)\) and Adolescents made more voluntary retreats than did Adults \((F_{1,30} = 4.42, p = 0.044)\). Other main effects and interactions were non-significant \((p > 0.12)\). See Figure 2-7 (b), (c) and (d).
Figure 2-2. Mean (± S.E.M.) of (a, b) latency to start drinking EtOH, (c, d) time spent drinking EtOH and (e, f) time at the mesh for CTL (control) and SS (adolescent social instability stress) male rats tested in (A) adolescence or in (B) adulthood. * indicates significant effect of Stress group, # indicates significant effect of Social condition.
**Phase I, Expt 2: Sucrose intake and social context**

(A) ADOLESCENTS

(a) Latency to Drink Sucrose

(b) Latency to Drink Sucrose

(c) Intake of Sucrose

(d) Intake of Sucrose

(e) Time at the mesh

(f) Time at the mesh

Figure 2-3. Mean (± S.E.M.) of (a, b) latency to start drinking Sucrose, (c, d) time spent drinking Sucrose and (e, f) time at the mesh for CTL (control) and SS (adolescent social instability stress) male rats tested in (A) adolescence or in (B) adulthood. # indicates significant effect of Social condition.
Figure 2-4. Mean (± S.E.M.) of (a) latency to start drinking and (c) time spent drinking EtOH and (b) latency to start drinking and (d) time spent drinking Water for CTL (control) and SS (adolescent social instability stress) male rats tested in adolescence or in adulthood. * indicates significant effect of Stress group, # indicates significant effect of Age. Significant effect of Test day in (a) (b) and (c) not shown.
Figure 2-5. Mean (± S.E.M.) of (a) latency to start drinking and (c) time spent drinking Sucrose and (b) latency to start drinking and (d) time spent drinking Water for CTL (control) and SS (adolescent social instability stress) male rats tested in adolescence or in adulthood. * indicates significant effect of Stress group, # indicates significant effect of Age. Significant effect of Test day in (a) (b) and (d) not shown.
Phase III, Expt 1: Competition for EtOH against cage partner

**Figure 2-6.** Mean (± S.E.M.) of (a) time spent drinking EtOH, (b) voluntary retreats, (c) number of face whacks, and (d) number of involuntary retreats for pairs of CTL (control) and SS (adolescent social instability stress) male rats tested in adolescence or in adulthood. * indicates significant effect of Stress group, # indicates significant effect of Age. Significant effect of Test day in (a) (c) and (d) not shown.
Figure 2-7. Mean (± S.E.M.) of (a) time spent drinking Sucrose, (b) voluntary retreats, (c) number of face whacks, and (d) number of involuntary retreats for pairs of CTL (control) and SS (adolescent social instability stress) male rats tested in adolescence or in adulthood. * indicates significant effect of Stress group, # indicates significant effect of Age. Significant effect of Test day in (a) (b) and (c) not shown.
4. Discussion

We tested the hypothesis that SS rats would show increased intake of ethanol relative to CTL rats when tested either soon after the adolescent SS exposure or long after the exposure in adulthood. Further, we hypothesized that the effect of SS would be moderated by social context. Consistent with the first hypothesis, SS rats exhibited an increase in the voluntary consumption of ethanol, but inconsistent with the second hypothesis, the higher intake of SS relative to control rats was evident irrespective of social context – when alone, in the presence of an unfamiliar peer, in the presence of the familiar cage partner, or when competing for access to ethanol against the familiar cage partner. Overall the differences between CTL and SS rats in ethanol intake were driven by those tested in adolescence rather than in adulthood. In a separate set of the same experiments, we compared SS and control rats on the intake of 1% sucrose and found a higher intake in SS rats than in CTL rats irrespective of age and only in the competition for access task (Phase III).

In this study, rats spent relatively little time drinking ethanol compared with the time spent drinking sucrose and had shorter latencies to drink sucrose than ethanol. Although these involved separate experiments, the differences between ethanol and sucrose are evident in the figures. Also, results provide evidence of unsweetened ethanol’s low palatability: in Phase II, time spent drinking ethanol decreased from the first to last test day, whereas rats drinking sucrose increased their intake across days. Nevertheless, ethanol was not aversive; rats did voluntarily consume ethanol, and when they had to compete for access to ethanol (Phase III), their intake increased across days. Differential sensitivity to reward between age groups and the degree of palatability/reward value of the two drinks may be critical factors in the higher intake of ethanol in SS rats compared with CTL rats primarily in adolescence and the higher intake of
sucrose in SS rats compared with CTL rats irrespective of age and only in the competition context. In both Phase II and III, the intake of sucrose was greater for adolescents than for adults, consistent with the finding of greater intake of sweetened condensed milk at PND 50 than in adults (Friemel et al., 2010), whereas an age difference was only when they had to compete for access to ethanol (Phase III). Thus, it may be that the low palatability of ethanol is sufficient to obtain effects of SS only in adolescence because it is a time of peak sensitivity to reward, whereas for more palatable substances, such as sucrose, the high palatability leads to effects of SS that are only observable under conditions of competition.

Most studies of chronic stress in adults that involve sucrose intake have used sucrose preference as a measure of anhedonia, with the expectation than sucrose intake would be diminished, based on evidence that chronic stressors induce depressive-like behaviour (Grippo, Beltz, & Johnson, 2003; Konkle et al., 2003; Sobrian, Marr, & Ressman, 2003; Willner, Muscat, & Papp, 1992). The extent to which adolescent chronic stressors reduces or increases sucrose intake depends on the procedure and the length of time after stress exposures the rats are tested (reviewed in McCormick & Green, 2013). For example, social isolation in adolescence from PND 30 to 50 did not affect sucrose intake in adult male rats and increased sucrose intake in adult female rats (Hong et al., 2012). Female rats that underwent chronic unpredictable stress from PND 23 to 51 decreased consumption of sucrose in adulthood, without any effect in male rats (Pohl, Olmstead, Wynne-Edwards, Harkness, & Menard, 2007). A mixed stressor procedure (isolation and social defeat) from PND 36 to 48 induced a decrease in sucrose consumption when tested one day after the termination of stress in both males and females (Harrell, Hardy, Boss-Williams, Weiss, & Neigh, 2013). Our finding of increased sucrose intake in adult males after adolescent SS only in the competition task suggests sucrose only modestly increased hedonic
value, perhaps because of the milder nature of the stress procedure. Notably, we previously reported no increase in depressive-like behaviour (immobility; passive coping) as measured in the forced swim test in adult males after adolescent SS (Mathews, Wilton, Styles, & McCormick, 2008). Instead, SS males showed more climbing than did CTL males, which has been termed “active coping.”

The finding of greater intake in SS than in CTL rats in the competition task is consistent with our previous report of greater intake of sweetened condensed milk in SS rats than in CTL rats when tested as adults (Cumming et al., 2014). In contrast to that report, however, we did not find SS rats to show more aggression (face whacks) than CTL rats during competition and the number of face whacks observed in Phase III, Experiment 2 were about 5-fold fewer in the 5th session than in (Cumming et al., 2014). The difference may be related to the higher palatability of sweetened condensed milk (which contains about 30% sucrose); pairs of rats in that study spent ~100 seconds at the feeder on the 5th day, in contrast to ~50 seconds in the adult pairs drinking 1% sucrose and ~8 seconds in the adult pairs drinking 10% ethanol in the present study. The lower palatability of the drinks in the present study may also be the reason why no consistent dominant-submissive relationships (whereby one of the pair drinks more consistently than the other) were evident in the competition. Also, the increased aggression in SS rats when competing for sweetened condensed milk was driven by the competitive situation, since they are not aggressive during the SS procedure. Although SS rats did not exhibit more aggression during competition here, that intake, face whacks, and involuntary retreats increased from the 1st to 5th session for both ethanol and sucrose is consistent with our previous report. Overall, adolescents had a greater intake than did adults in the competitions in Phase III, which is consistent with
evidence of a higher sensitivity to rewards in adolescence (reviewed in Doremus-Fitzwater & Spear, 2016).

The higher sensitivity to reward in adolescents than in adults has been linked to changes in the neural circuitry mediating reward. During the adolescent period, there is an increase in dopamine innervations, activity, and number of receptors in the prefrontal cortex, striatum and nucleus accumbens (NAc), which peaks after puberty and plateaus in adulthood (reviewed in Burke & Miczek, 2014). This maturation process can be influenced by stressors; the ventral tegmental area (VTA) receives projections from the paraventricular nucleus of hypothalamus and expresses corticotropin releasing hormone receptors (CRHR) and glucocorticoid receptors (GR) (reviewed in Ungless, Argilli, & Bonci, 2010). Stressful events can increase dopamine release from the VTA via increases in CRH release, and this effect is extinguished by CRH antagonist infusion in the VTA (Wang et al., 2005). Glucocorticoids also modulate VTA activity: the reduction of GR in dopamine-innervated neurons in the NAc and striatum in mice reduced their motivation to self-administer cocaine (Ambroggi et al., 2009), and impaired conditioned place preference (CPP) and locomotor sensitization to low dose of amphetamine (Parnaudeau et al., 2014). Rats that underwent the SS procedure in adolescence showed increased locomotor sensitization to amphetamine when tested in either adolescence or in adulthood (Mathews, Mills, et al., 2008). Thus, the higher intake of SS than CTL rats may be attributable to changes in the mesocorticolimbic circuitry resulting from a greater exposure to glucocorticoids during the SS procedure when these systems are maturing.

One possibility is that the consistently greater intake of ethanol in SS rats than in control rats is based in the anxiolytic properties of ethanol rather than its reward value. Adolescent rats that were restrained for five days show reduced social interactions, but when exposed to ethanol,
stressed rats increased social interaction, whereas ethanol reduced social interaction in non-stressed rats (Varlinskaya, Truxell, & Spear, 2013). This study, however, involved sweetened ethanol, and there was no difference in intake between the stressed and non-stressed rats. We have some evidence of increased anxiety in SS rats based on SS male rats spending less time spent in the open arms of an elevated plus maze (McCormick et al., 2008), as well as by an increased latency to enter the centre of an open field and in addition to reduced time in social interaction with an unfamiliar peer (Green et al., 2013). Another possibility is that differences in the drinking of ethanol and sucrose of SS rats compared to CTL rats is because the stress experience modified the sensitivity to tastes. The only evidence we know for such a possibility is that neonatal handling decreased positive reactions in response to sucrose compared to control rats tested by taste reactivity in adulthood (Silveira et al., 2010). Whether SS in adolescence modifies taste sensitivity remains to be determined.

We had predicted that CTL rats, and possibly SS rats, would spend more time drinking EtOH in the presence of a peer than when alone based on Varlinskaya and colleagues (Varlinskaya et al., 2015a). Our lack of a difference between dinking alone or with a peer is likely because of methodological differences. First, our rats were unfamiliar to each other and kept apart by a wire mesh, whereas in the Varlinskaya study, rats were tested in groups of 4 to 5 littermates without any physical separation. We opted for a wire mesh because SS rats do not engage in as much social interaction as do CTL rats when physical contact is possible, but spend as much time (and sometimes more time) than do CTL rats near peers behind mesh (Green et al., 2013; Hodges et al., 2017). Second, the Varlinskaya study involved 6 drinking sessions alternating between the alone and social condition whereas our experiment involved one session only. In a study of social learning, adolescent male rats drink more ethanol after interacting with
a familiar demonstrator than after interacting with an unfamiliar one (Maldonado, Finkbeiner, & Kirstein, 2008). Thus, the exposure of rats to multiple sessions with their cagemates may have increased the amount of ethanol drank (Varlinskaya et al., 2015a). In addition, the experiments here involved an unsweetened ethanol solution, whereas in the Varlinskaya study, the ethanol solution was sweetened with sucrose and saccharin. In sum, any of these differences may have influenced the extent to which rats drank when alone versus when with peer(s). Our use of unsweetened ethanol may also be why no age difference was observed in the intake of ethanol. Nevertheless, although most studies finding greater intake in adolescent than in adult male rats involved sweetened ethanol (Broadwater et al., 2013; Doremus et al., 2005; Maldonado, Finkbeiner, Alipour, & Kirstein, 2008; Varlinskaya et al., 2015a; Varlinskaya, Truxell, & Spear, 2015b; Walker, Walker, & Ehlers, 2008), some have observed the age difference using unsweetened ethanol (Acevedo et al., 2016; Vetter et al., 2007, but also see Siegmund et al., 2005).

There have been relatively few studies of how stress in adolescence influences ethanol intake in rats (Acevedo et al., 2016; Brunell & Spear, 2005; Wille-Bille, Ferreyra, et al., 2017). The studies that have investigated social stressors effects mostly involved isolation housing. For example, a comparison of sweetened ethanol intake in adolescent (PND 23) and adult (PND 60) male rats that were either pair-housed or isolation-housed for 12 days, reported a decrease in intake after isolation in adults, though not in adolescents (Doremus et al., 2005), which may be related to the addition of sweeteners. In contrast, six weeks of isolation housing from PND 21 to 63 increased the intake of unsweetened 10% ethanol compared to group housed male rats (McCool & Chappell, 2009). Rats that were socially housed but were isolated for 12h/day every other day from PND 25 to 35 drank more unsweetened 8% ethanol than did control males
(Juárez & Vázquez-Cortés, 2003). Our findings are consistent with these studies and indicate that social instability can increase ethanol intake, as does social isolation housing. The present experiments show that the increased ethanol intake after social instability stress is evident across a variety of social contexts and that the differences may extend into adulthood. The next steps will be to identify the specific neural mechanisms, likely involving the mesocorticolicimbic dopamine system, that underlie these effects of adolescent social instability stress. Females typically show higher ethanol intake than males (Broadwater et al., 2013; Lancaster & Spiegel, 1992; Tambour, Brown, & Crabbe, 2008; Varlinskaya et al., 2015b; Wille-Bille, Ferreyra, et al., 2017, but also see Dhaher et al., 2012; Vetter-O’Hagen, Varlinskaya, & Spear, 2009). Thus, it will also be important to investigate the effects of SS on drinking in females. Although Roeckner and colleagues (2017) did not find an effect of SS in females on intake of 20% ethanol, SS females may show greater effects in the test procedures we used in this study. Further, for some drugs of abuse (nicotine and amphetamine), the effects of SS are also sex-specific (reviewed in McCormick, 2010).
Chapter 3: The effects of social instability stress and subsequent ethanol consumption in adolescence on brain and behavioural development in male rats

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Author contribution: for this manuscript, Dr Cheryl McCormick and I designed the experimental and formulated the research questions. I wrote the proposal for the ethics approval, ordered the rats required for the experiment, performed the social instability stress procedure on rats, performed the behavioural experiments (with help from Travis Hodges and Jennet Baumbach), scored videos, analyzed the data, performed the statistics (with help from Jennet Baumbach), I helped to create the figures, wrote the paper and edited after receiving feedback from Dr Cheryl McCormick.
1. Introduction

Alcohol abuse and dependence are serious health problems in developed nations. In Canada, alcohol is the most consumed drug and it caused more hospitalizations than did heart diseases in 2015-2016 (https://www.ccsa.ca/alcohol-canadian-drug-summary). According to a survey in 2015, over 75% of Canadians 15 years old or older had consumed alcohol at least once in the previous year. The prevalence of alcohol use in the past 12 months among teenagers (12-17 years) has decreased from 45.4% in 2010-2011 to 39.5% in 2014-2015, and the average onset age of drinking was 13.5 years. The prevalence of high schoolers consuming five or more drinks in one occasion (defined as binge drinking) has also been decreasing over the years, reaching 33.3% in 2010-2011 and 23.7% in 2014-2015. Binge drinking is defined as reaching blood alcohol concentrations (BAC) greater than 80 mg/dl (or 0.08 %) in about two hours, which is achieved by ingesting 4 or more drinks for women and 5 or more drinks for men. One drink is defined as 341 ml or 12 oz of beer of 5% alcohol content, 142 ml or 5 oz of wine of 12% alcohol content, or 43 ml or 1.5 oz of distilled beverages of 40% alcohol content (https://www.niaaa.nih.gov/alcohol-health/overview-alcohol-consumption/what-standard-drink).

Even though the prevalence of adolescent alcohol consumption has been decreasing over the years, the numbers are still high and the average onset age of drinking is at 13.5 years.

These data indicate a great risk for teenagers to develop problems with alcohol addiction; the earlier the onset of drinking, the greater the risk of becoming dependent on alcohol (Bonomo et al., 2004). In humans, exposure to illicit drugs or alcohol in the teen years increases the risk of negative consequences in adulthood (Odgers et al., 2008) relative to when use starts in adulthood (Clark, Kirisci, & Tarter, 1998). Higher risk of alcohol dependence when alcohol intake begins during the adolescent period can also be found in rodent models of drinking, but the results are
mixed: some studies find rats exposed to ethanol during adolescence show increased ethanol consumption in adulthood (e.g. Maldonado-Devincci et al., 2010), whereas other studies found no increase (e.g. Gilpin, Karanikas, & Richardson, 2012). Adolescent rats, however, consume more ethanol per body weight than do their adult counterparts (Doremus et al., 2005), which may be driven by differences in the subjective reward value of ethanol. Adolescent rodents also are more sensitive to ethanol’s positive effects and less sensitive to its negative effects than are adults (Spear & Varlinskaya, 2010).

Animal models of binge drinking aim to reach or surpass the 80 mg/dl of blood alcohol content (BAC) within 30 to 60 minutes of drinking, which may involve repeated exposures for a few days to a few months. There are many different protocols used to administer ethanol to rodents, but the most common ones are intraperitoneal injection (i.p.), ethanol vapor inhalation, oral intubation (or gavage, i.g.), and voluntary consumption of ethanol solutions that can be sweetened or not (reviewed in Bailey, Gerlai, Cameron, Marcolin, & McCormick, under review). The protocol used in the present study was the Intermittent Access to 2-bottle choice (IA2BC), which is a reliable method to induce high ethanol intake by rats in a voluntary way without water deprivation, and results in greater consumption than does continuous access (Carnicella, Ron, & Barak, 2014). Adult rats typically consume stable, high levels of ethanol (>4.5 g/kg/24 h) after 3-4 weeks of exposure (Simms et al., 2008). There is a growing literature on adolescent ethanol exposure using the IA2BC procedure, which usually finds that adolescent rats consume more than do adults (Dhaher et al., 2012; Fernandez et al., 2016) although there are exceptions (Wille-Bille, de Olmos, et al., 2017).

In rats, exposure to ethanol in adolescence has the potential to influence ongoing brain and behavioural development. For example, ethanol consumption through voluntary drinking
during adolescence can induce anhedonia (measured as lower sucrose preference) and depression-like symptoms (measured as increased time immobile on forced swim test) (Briones & Woods, 2013), anxiety-like symptoms (measured as reduced time in the open arm on the Elevated Plus Maze) (Marco et al., 2017), decreased expression of genes in the periaqueductal gray that are involved in fear and anxiety (McClintick et al., 2016), as well as decreases in genes and pathways involved in neurogenesis, long-term potentiation, and axonal guidance in the ventral hippocampus, which could relate to the impaired memory function found in subjects with adolescent ethanol binge-like exposure (McClintick et al., 2018). Ethanol exposure can also increase baseline corticosterone concentrations (Cippitelli et al., 2014). Thus, understanding factors that promote ethanol intake in adolescence is of importance.

Stressors, particularly early life stressors, are a known risk factor for alcohol use disorders in humans (Enoch, 2011). In rodents, stressors in adolescence can also increase ethanol intake (e.g. Wille-Bille, Ferreyra, et al., 2017), although some have reported that stressors reduce or do not alter ethanol intake in adults (Brunell & Spear, 2005; Thorsell et al., 2005). Adolescence is a time of social learning and of ongoing development of the social brain (McCormick et al., 2015). Thus, adolescents may be particularly susceptible to social stressors. Social isolation has been used as a stressor in studies of ethanol exposure (Butler, Karkhanis, Jones, & Weiner, 2016). During adolescence, social isolation can increase voluntary consumption of ethanol in rats and mice tested in adulthood (Butler, Carter, et al., 2014; Chappell et al., 2013; Lesscher et al., 2015; Schenk et al., 1990; Skelly et al., 2015). Social defeat is another social stressor commonly used in rodent studies, whereby intruders are subjugated by aggressive residents that are defending their territory (reviewed in Newman, Leonard, Arena, de Almeida, & Miczek, 2018). Social defeat typically induces a depressive-like
state in the intruding rodents, although both decreases (Funk, Harding, Juzytsch, & Lê, 2005; van Erp & Miczek, 2001; van Erp, Tachi, & Miczek, 2001) and increases in ethanol intake (Caldwell & Riccio, 2010; Funk, Vohra, & Lê, 2004) were reported in adults after social defeat. Very few studies have tested the effects of social defeat in adolescent rodents. The available data indicate that ethanol intake and willingness to work for ethanol remained increased relative to control rats for weeks after adolescent social defeat (Riga et al., 2014; Rodriguez-Arias et al., 2016).

Our model of social instability stress (SS) constitutes a relatively mild social stressor relative to social defeat or prolonged isolation, and adolescents are more sensitive to the lasting consequences of SS than are adults (Hodges & McCormick, 2015; McCormick et al., 2004). The SS procedure consists of daily one-hour isolation after which rats are housed with a new cage partner that also is undergoing daily isolation from postnatal day (PND) 30 to 45. SS in adolescence leads to altered social behaviour, notably a reduction in social interaction without a reduction in (and perhaps enhanced) social motivation (Green et al., 2013; Hodges et al., 2017). SS rats also show enhanced motivation for sweetened drinks under conditions of competition (Cumming et al., 2014; Marcolin, Hodges, Baumbach, & McCormick, 2019), and increased locomotor sensitization to psychostimulants such as amphetamine and nicotine (Mathews, Mills, et al., 2008; McCormick & Ibrahim, 2007; McCormick et al., 2004, 2005). Thus, SS rats may differ from control rats in motivation for, and responsivity to, reward. The psychostimulant studies, however, were limited by the fact that drugs were administered to the rats, and thus do not address how readily SS rats would self-administer the drugs. This limitation can be addressed using ethanol, which rats voluntarily consume. Studies in which animals choose to ingest ethanol are more appropriate than experimenter-administered ethanol to assess motivational aspects of ethanol intake because they produce changes in brain self-stimulating reward parameters that are
not induced by the latter (Moolten & Kornetsky, 1990). We have found that SS rats drink more ethanol than do control rats in a variety of social contexts during adolescence (Marcolin et al., 2019), however we have not investigated whether SS in adolescence increases the propensity to escalate ethanol consumption.

In this study, we aimed to answer two main questions: 1) Does the history of adolescent social stress increase the propensity to escalate ethanol consumption? 2) Are the negative consequences of chronic intermittent ethanol drinking greater for SS rats than for control rats as measured by social and anxiety-like behaviour, and relevant physiological correlates (corticosterone concentrations and neuronal measures)? We tested voluntary ethanol intake using the IA2BC procedure in SS and control rats beginning at the end of the SS exposures in mid-adolescence to early adulthood. In experiment 1, we evaluated neuronal measurements such as markers of synaptic plasticity (spinophilin, postsynaptic density protein 95 [PSD95], and calcium/calmodulin-dependent protein kinase II [CaMKII]), glucocorticoid receptor and dopamine receptors 1 and 2 in regions involved with reward, such as the nucleus accumbens (nAcc), prefrontal cortex (PFC) and hippocampus (Juarez & Han, 2016). In experiment 2, we evaluated behavioural measurements that have been shown to be sensitive to ethanol drinking such as anxiety, social interaction, and memory (Spear, 2018), as well as the intake of ethanol after a period of withdrawal.

2. Materials and Methods

2.1. Social Instability Stress

Male Long-Evans rats (n = 76, experiment 1; n = 100, experiment 2) were purchased from Charles River (USA) and arrived on PND 23. Rats were randomly pair-housed in 30 cm x
33 cm x 18 cm home cages with free access to food and water. The colony room was on a 12:12 h light cycle (lights on at 6:00 h). Rats were left undisturbed until PND 30, when half of the rats underwent the social instability stress (SS) procedure, (reviewed in (McCormick, 2010). Briefly, SS rats were isolated in a ventilated container (12 cm x 10 cm) for one hour daily from PND 30-45. After the isolation, rats returned to a new cage partner every day also undergoing daily isolation, never meeting the same rat twice. After the 16th isolation, rats returned to their original cage partner. SS was performed during the light cycle at a different time on each day to minimize habituation to the procedure. Non-stressed control (CTL) rats were left undisturbed except for regular cage maintenance from PND 30-45. All procedures were approved by the Brock University Institutional Animal Care Committee (ACC) and were carried out in adherence to the Canadian Council on Animal Care guidelines. See Figure 3-1 for the design and procedures of the two experiments.

2.2. Chronic Intermittent Drinking Model

For experiment 1, SS and CTL rats had 24-hour access to two bottles in their home cage every Monday, Wednesday and Friday between PND 47-66 (9 days of 24-hour access starting at 18:00 h). One of the bottles contained tap water and the second bottle contained either tap water (control condition) or a 10% ethanol (EtOH) solution sweetened with 0.1% saccharin (chronic intermittent access condition). EtOH was prepared fresh each day by diluting 95% EtOH with the appropriate volume of tap water. During the remaining days of the week, two bottles of tap water were available in all cages. The bottles were weighed before and after the 24-hour access periods, and the position of the two bottles within a cage was switched every time to avoid influences of side biases on intake. The use of saccharin serves two purposes: first, to help overcome EtOH’s initial aversive taste and to increase the palatability of the solution without
changing caloric content, and second, to mimic the sweet-alcoholic solutions that teenagers usually start consuming (Vendruscolo et al., 2010).

Rats were weighed on PND 46 and at the end of each week and a linear regression model was used to estimate the body weight at each day. The body weight (BW) of the cage partners was averaged to express the consumption of solutions per cage. Although many studies of EtOH intake house rats singly so that each individual’s consumption can be accurately determined, we did not want to introduce stress of single housing as a confound. The consumption was calculated as the bottle weight difference between the start and the end of the sessions, minus the amount of spillage and/or evaporation of bottles placed in an empty cage (usually 1-3 grams/day) and divided by the average body weight of the cage, providing the consumption in grams of solution/kg BW. For the EtOH consumption, this number was multiplied by 0.08589 for the weight of EtOH in 1 g of a 10% v/v EtOH solution, giving the consumption in grams of EtOH/kg BW. The average consumption across the three days of access for each of the three weeks for each bottle was used for data analysis.

For experiment 2, a second IA2BC condition was added to the study: 0.1% saccharin without any EtOH, to control for any effects that the consumption of saccharin might have. In a pilot experiment (data not shown), rats consumed entire bottles of saccharin (200 ml) during 24 hours of access, which resulted in greater saccharin intake than in the EtOH group. Therefore, the purpose of the saccharin control in the second experiment was to match the saccharin consumption between the two solution groups. We calculated the amount of saccharin in grams/kg BW that was consumed by the EtOH group in experiment 1 and gave the rats in experiment 2 the appropriate volume of water containing the same amount of saccharin.
Figure 3-1. Experimental design and procedures. PND = postnatal day, IA2BC = Intermittent Access to 2-Bottle Choice model, EtOH = ethanol, nAcc = nucleus accumbens, PFC = prefrontal cortex, VH = ventral hippocampus, DH = dorsal hippocampus, CaMKII = calcium/calmodulin-dependent protein kinase II, D1 = dopamine receptor 1, D2 = dopamine receptor 2, GR = glucocorticoid receptor.

2.3. Experiment 1: Neural measures

On PND 70, four days after the last EtOH access period, brains were collected by rapid decapitation, fast-frozen in isopentane on dry ice and stored at -80°C until Western blot analyses. Another subset of CTL and SS rats (n = 8/group) on PND 46 was decapitated the same way as adults for an adolescent comparison. Brains were sliced into 1 mm thick slices using a pre-chilled brain mould on ice and razor blades. Slices were placed onto a chilled petri dish and brain regions were collected bi-laterally using a 1.0 mm diameter tissue punch. Punches of the nucleus
accumbens (nAcc, +1.92 mm to +0.96 mm, identified according to Paxinos and Watson, 2005), prefrontal cortex (PFC, +3.72mm to + 2.76 mm), ventral hippocampus (VH, -4.56 to -5.76 mm) and dorsal hippocampus (DH, -2.16 to -4.20 mm) were dissected and homogenized in cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% Triton-X, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 2 mM Na₃VO₄ and 50 mM NaF). These brain regions were selected because they integrate the system that mediates reward and reinforcement of drugs of abuse (reviewed in You et al., 2018). We investigated the dorsal and ventral subregions of the hippocampus separately given their functional differences (e.g. (Fanselow & Dong, 2010; Thompson et al., 2008), which may render the two regions different in sensitivity to the manipulations. Samples were sonicated using 0.5 mm glass beads (NextAdvance, USA) in a Bullet Blender® (NextAdvance, USA) for 3 min, incubated on ice for 25 min, centrifuged at 16,000 x g for 10 min at 4°C. The supernatant was collected and used to quantify protein concentration via Bradford assay (Bio-Rad). Samples were brought to same protein concentration using lysis buffer without proteases inhibitors, diluted with Laemmli buffer 2X, heated at 70°C for 5 min, centrifuged at 16,000 x g for 10 min at 4°C and stored at -20°C until use.

Prepared samples were heated for 5 min at 70 °C, vortexed, and loaded (7.5 or 10 μg/sample) onto 12% polyacrylamide TGX Stain-Free fast-cast (Bio-Rad, USA) and electrophoresis was run at 250V for 30 min. Gels were exposed to UV light for 5 min for activation of stain-free total protein determination. Proteins were transferred to a PVDF membrane (Millipore) at 100V for 30 min. Membranes were washed 3x for 5 min with TBS-T, blocked with 5% milk (for GR) or BSA (for the others) dissolved in TBS-T buffer for 1 hour under agitation at room temperature and incubated overnight under agitation at 4°C with primary antibodies. Membranes were washed 3x for 5 min with TBS-T and incubated with fluorophore-conjugated secondary
antibodies (Alexa647 anti-mouse and Alexa488 anti-rabbit, 1:5,000 each; Life Technologies, USA) for 45 min under agitation at room temperature. Membranes were washed 3x for 5 min with TBS-T and images were obtained using Bio-Rad VersaDoc MP 4,000 Imaging System (Bio-Rad, USA) and the Quantity One VersaDoc Imaging Software. Bands were quantified using Bio-Rad Image Lab software version 4.1 (Bio-Rad, USA). The density of each band was normalized to total protein using the TGX Stain-Free total protein determination method (Bio-Rad, USA), and individual values were then normalized relative to the mean value of the controls, which was set to 100%. Each experimental group was equally represented and counter-balanced across each gel. Primary antibodies used: anti-GR 1:1,000 #sc-1004 Santa Cruz Biotechnologies; anti-D1 1:200 #sc-14001 Santa Cruz Biotechnologies; anti-D2 1:500 #sc-9113 Santa Cruz Biotechnologies; anti-spinophilin 1:5,000 #06-852, Millipore; anti-PSD-95 1:5,000 #NB300-556 Novus Biologicals; anti-CaMKII 1:10,000 #04-1079 Millipore.

2.4. Experiment 2: Behavioural measures

2.4.1. Elevated Plus Maze (EPM)

On PND 70 or 71, rats were tested for anxiety-related behaviour in the Elevated Plus Maze apparatus under dim illumination starting at 9:00 h (3 hours after lights on). The apparatus was elevated 80 cm above the ground, the arms were 50 cm in length each, and walls of the closed arm were 42 cm high. The open arms had 1.3 cm ledges around the edge of the platform. Each rat was individually placed on the center of the apparatus facing the closed arm, and was left alone to explore the EPM for 5 minutes. EPM behaviour was recorded by a camera mounted to the ceiling, and the number of entries and time spent in each arm, rearing behaviour (in the
closed arm), and head dipping (in the open arm) were scored by a researcher blind to the experimental groups.

2.4.2. Social Interaction

Several hours after EPM testing (starting at 19:00 h, one hour after lights off), rats were tested for social interaction with an unfamiliar rat from the same experimental condition. Pairs of unfamiliar rats from the same experimental group were placed in an apparatus (60 x 60 x 60 cm) under dim red light, and social interactions between the rats were recorded by a camera mounted to the ceiling for 15 min. Social interaction was defined as time spent in physical contact initiated by each of the two rats (Green et al., 2013; Hodges et al., 2017). Time spent in social interaction was quantified by an experimenter blind to the experimental groups.

2.4.3. Contextual Fear Conditioning and Fear Recall

On PND 72 or 73, starting at 10:00 h (4 hours after lights on), rats were transported from the colony room to a separate behavioural testing room in their home cages on a metal cart where 4 fear conditioning chambers (30 x 37 x 25 cm) were situated in a larger, ventilated sound-attenuating chambers. The outer chambers consisted of a small window (8 cm diameter) on the door that was covered by a red Plexiglas® pane (all equipment from PanLab, Spain). The inner chamber rear and side walls were made of black stainless steel, and the door of clear Plexiglas®. The floor consisted of 20 stainless steel rods wired to a shock source and a solid-state scrambler that allowed for the delivery of the unconditioned stimulus, a 0.5 mA, 1-second foot shock. The grid floor rested approximately 1 cm above a black stainless-steel base that was placed on weight transducers that connected to a load cell coupler designed to detect fine movement. Rats were
allowed to explore the chambers for a 3 minute habituation period before the presentation of three 1s foot shocks spaced 1 minute apart. Rats remained in the chamber for 1 min after the last shock (6 minutes total in the chamber). Automated software (Freezing, PanLab) displayed the percentage of time spent freezing for each rat during the first 3 minutes and the second 3 minutes in the chamber. To record a freezing event (the measure of fear), the load cell coupler weight transducer required activity to be below the activity threshold for a minimum of 3000 ms. On PND 73 or 74, rats were tested for contextual fear recall (time spent freezing) starting at 13:00 h (7 hours after lights on). Rats were transported back to the behavioural testing room in their home cages on a metal cart and placed into the same conditioning chambers for a 20-minute period without shock.

2.4.4. Plasma Corticosterone Determination

Blood samples (approximately 200 µl) were obtained at different time points from a tail nick and collected into ice-chilled tubes containing EDTA (Microvette® CB 300 K2E, Sarstedt, Germany) within 2 minutes. The time points were: a) baseline (before fear conditioning), b) post-fear conditioning (20 min after being placed into the conditioning chambers), c) immediately after fear recall, and d) 30 min after the end of fear recall. Samples were centrifuged at 1,730 x g for 10 min at 4°C. Plasma was collected and frozen at -20°C until the time of assay. Steroids were extracted mixing 25-50 µl of plasma with 2 ml of diethyl ether. Plasma samples were reconstituted in buffer provided in the enzyme-linked immunosorbent assay kits for the measurement of corticosterone (#402810 Neogen, Lansing MI). The assay was conducted according to the kit instructions and quantified using a Biotek Synergy plate reader. Assay
sensitivity was 0.05 µg/mL, and intra- and inter-assay variance were less than 10% and 15%, respectively.

2.4.5. Ethanol Intake After Withdrawal

On PND 75, all rats were given one bottle with 10% EtOH solution sweetened with 0.1% saccharin and one bottle with water in their home cage, to measure the differential EtOH consumption between rats that were never exposed to EtOH and rats that had previous chronic intermittent access to EtOH. The bottles were weighed before and after the first hour and again after 24 hours to calculate grams consumed per cage (divided by 2 to estimate consumption per rat). This measure was discounted by the weight of control bottles placed in an empty cage containing the same solutions tested, to control for dripping and evaporation. Rats were weighed before the EtOH bottle was introduced and 24 hours later, and the body weight of the cage partners was averaged to express the consumption per cage as g of EtOH consumed/kg BW.

2.5. Statistical Analysis

Statistical analyses were performed using SPSS version 25 software and consisted of analyses of variance (ANOVA) or repeated measures ANOVA, where appropriate. Alpha was set at $p < 0.05$. Post-hoc analyses consisted of paired-samples or independent-samples $t$-test.
3. Results

3.1. Experiment 1

3.1.1. Ethanol Drinking

Analyses for the chronic intermittent drinking data involved Week (Week 1, 2, 3) X Bottle (Water x Water/EtOH) X Stress Group (CTL x SS) ANOVAs for each separate drinking group.

Water Group: There was a main effect of Week ($F_{2,28} = 58.72$, $p < 0.0001$), with a decline in intake across weeks. SS rats drank more water than did CTL rats ($F_{1,14} = 4.67$, $p = 0.049$), and there was no interaction of Stress Group with any other factor (all $p > 0.10$). See Figure 3-2(a).

EtOH Group: There was an interaction of Week and Bottle ($F_{2,40} = 53.42$, $p < 0.0001$); EtOH intake increased, whereas water intake decreased, across weeks. The increased overall intake of SS rats than CTL rats was not significant ($F_{1,20} = 4.0$, $p = 0.06$), with no difference between SS and CTL for EtOH intake ($p = 0.79$) or for water intake ($p = 0.20$), and there was no interaction of Stress Group with any other factor (all $p > 0.40$). See Figure 3-2(b). There also was no difference between SS and CTL rats on grams of EtOH consumed over the weeks ($p = 0.79$), see Figure 3-2(c).

3.1.2. Protein Expression

PND 46: A one-way ANOVA indicated there were no Stress Group differences for any of the markers of synaptic plasticity or for dopamine receptors in either the PFC or nAcc at PND 46 (all $p > 0.54$). All data means and standard error of the means are shown in Table 1.

Table 3-1. Summary of Western blot data for adolescent animals (PND 46). Data expressed as mean (SEM), $n=8$/group.
PND 70: A Stress Group (CTL x SS) X Solution Group (Water x EtOH) ANOVA indicated a main effect of Solution in the dorsal hippocampus for αCaMKII, βCaMKII and PSD-95 (F\(_{1,28} = 5.15, p = 0.031\), F\(_{1,28} = 4.38, p = 0.046\), and F\(_{1,28} = 8.41, p = 0.007\), respectively), with EtOH rats having lower expression than Water rats. There was also an interaction between Stress and Solution in the PFC for αCaMKII and PSD-95 (F\(_{1,25} = 4.35, p = 0.047\) and F\(_{1,25} = 12.4, p = 0.002\), respectively):

For αCaMKII, CTL EtOH rats had lower expression than CTL Water rats (p = 0.018), whereas SS Water and SS EtOH did not differ (p = 0.91); SS Water rats had lower expression than did CTL Water rats (p = 0.046), whereas CTL EtOH and SS EtOH did not differ (p = 0.68).

For PSD-95, CTL EtOH rats had lower expression than CTL Water rats (p = 0.005), whereas SS Water and SS EtOH did not differ (p = 0.19); SS Water rats had lower expression than did CTL Water rats (p = 0.037), and SS EtOH rats had greater expression than CTL EtOH rats (p = 0.009). All data means and standard error of the means are shown in Table 2.
### Table 3-2. Summary of Western blot data for adult animals (PND 70). Data expressed as mean (SEM), n=8/group. § CTL Water different from CTL EtOH, † CTL Water different from SS Water, ¥ CTL EtOH different from SS EtOH

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<td>(3.94)§</td>
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### 3.2. Experiment 2

#### 3.2.1. Ethanol Drinking

Analyses for the chronic intermittent drinking data involved Week (Week 1, 2, 3) X Bottle (Water x Water/EtOH) X Stress Group (CTL x SS) ANOVAs for each separate drinking group.

Water Group: There was an effect of Week ($F_{2,42} = 36.28, p < 0.001$), with a decline in intake across weeks. No other main effect or interaction was significant (all $p > 0.15$). See Figure 3-3(a).
Saccharin Group: There was an interaction of Week and Bottle ($F_{2,28} = 198.38, p < 0.001$); saccharin intake increased across weeks, whereas water intake decreased. There was no main effect of Stress Group ($p = 0.71$). Other than the main effect of Week ($p = 0.004$) and Bottle ($p < 0.001$), all other main effects and interactions were $p > 0.06$. See Figure 3-3(b).

EtOH Group: There was an interaction of Week and Bottle ($F_{2,28} = 35.46, p < 0.001$); EtOH intake increased, whereas water intake decreased, across weeks. There was an interaction of Bottle and Stress Group ($F_{1,14} = 6.42, p = 0.024$); SS rats drank less EtOH than did CTL rats ($p = 0.007$) and did not differ in water intake ($p = 0.20$), see Figure 3-3(c). Other than the main effect of Week ($p < 0.001$), all other main effects and interactions were $p > 0.10$. SS rats also consumed fewer grams of EtOH over the weeks than did CTL rats ($p = 0.007$), see Figure 3-3(d).

3.2.2. Elevated Plus Maze (EPM)

A Stress Group (CTL x SS) X Solution Group (Water x EtOH x Saccharin) ANOVA showed that SS rats spent more time on the open arm than did CTL rats but the difference did not meet statistical significance ($F_{1,94} = 3.02, p = 0.08$), nor did the main effect of Solution ($p = 0.79$) and interaction ($p = 0.41$). SS rats spent more time in the centre ($p = 0.008$) and less time in the closed arms ($p = 0.006$) than did CTL rats (other main effects and interactions $p > 0.15$). There were no main effects or interactions on the number of rears (all $p > 0.20$), and SS rats made more head dips than did CTL rats ($p = 0.041$; other main effect and interaction, $p > 0.20$). There was no main effect of Stress Group, Solution Group, or interaction on entries into the closed arms (all $p > 0.40$), indicating that group differences on anxiety-like measures were not confounded by differences in locomotor activity. See Figure 3-4.
3.2.3. Social Interaction

A Stress Group (CTL x SS) X Solution Group (Water x EtOH x Saccharin) ANOVA indicated a main effect of Solution Group on time spent in social interaction ($F_{2,94} = 4.68, p = 0.012$), whereby the EtOH rats spent less time in social interaction than did the Saccharin ($p = 0.019$) and Water ($p = 0.004$) rats, which did not differ ($p = 0.50$). The decreased time in social interaction of SS rats relative to CTL rats was as predicted based on our previous reports (Green et al., 2013; Hodges et al., 2017), but did not meet statistical significance ($p = 0.07$), nor did the interaction of Stress Group and Solution Group ($p = 0.24$). Nevertheless, separate ANOVAs in each of the Stress Groups suggested that the effect of Solution Group was driven by the reduction of social interaction in CTL rats of the EtOH Group (Effect of Solution Group in CTL rats, $p = 0.008$: EtOH vs Saccharin, $p = 0.006$ and EtOH vs Water, $p = 0.005$ and Water vs Saccharin $p = 0.74$. Effect of Solution Group in SS rats, $p = 0.58$). See Figure 3-5.

3.2.4. Contextual Fear Conditioning and Fear Recall

There were no Group differences or interaction of Solution Group and Stress Group on freezing during exploration (all $p > 0.34$), in freezing during fear conditioning (all $p > 0.23$), in freezing during fear recall (all $p > 0.24$). A Solution Group X Stress Group X Extinction Interval ANOVA found a decline in freezing across time intervals ($F_{2,188} = 74.46, p < 0.0001$). All remaining main effects and interactions were $p > 0.34$, except for the interaction of Stress Group and Extinction Interval ($p = 0.06$). See Figure 3-6.
3.2.5. Corticosterone Pre and Post Fear Conditioning and Recall

For some samples, not enough blood was collected for analysis within the two-minute allotment. Thus, analyses were conducted for each time point separately to allow the maximal number of samples in analyses. At baseline, the SS rats had higher corticosterone concentrations than did CTL rats ($F_{1,78} = 5.11, p = 0.027$), and there was an effect of Solution Group ($F_{2,78} = 10.51, p < 0.001$), whereby EtOH rats had lower corticosterone concentrations than did Saccharin ($p < 0.001$) and Water ($p = 0.003$) rats, and Saccharin rats had higher corticosterone concentrations than did Water rats ($p = 0.046$) The interaction of Stress Group and Solution Group was not significant ($p = 0.06$). After fear conditioning, no main effect or interaction was significant (all $p > 0.10$). Figure 3-7.

After fear recall, SS rats had lower corticosterone concentrations than did CTL rats ($F_{1,91} = 7.45, p = 0.008$; other main effect and interaction, $p > 0.65$). The lower corticosterone concentrations of SS rats than of CTL rats remained after 30 min of recovery ($F_{1,81}= 15.27, p < 0.001$), at which point EtOH rats had lower corticosterone concentrations than did Saccharin ($p = 0.013$) and Water ($p = 0.039$) rats, which did not differ ($p = 0.62$). Figure 3-7.

3.2.6. 1 hour of EtOH Intake (PND 75)

A Bottle (EtOH vs Water) x Stress Group (CTL x SS) X Solution Group (Water x EtOH x Saccharin) ANOVA resulted in a significant three-way interaction for solution intake ($F_{2,44} = 8.69, p = 0.001$). For the EtOH Group, SS drank less EtOH ($p = 0.041$) than did CTL rats and did not differ in water intake ($p = 0.08$). For the Saccharin Group, SS and CTL rats did not differ in water or EtOH intake (all $p > 0.16$). For the Water Group, SS drank more EtOH ($p = 0.012$) than did CTL rats, but did not differ in water intake ($p = 0.16$). Among CTL rats, the Water Group
drank more water than did the other two groups (both $p < 0.001$), which did not differ between them ($p = 0.88$); and the intake of EtOH was less than in the other two groups (both $p < 0.001$), which did not differ between them ($p = 0.28$). Among SS rats, the Solution Groups did not differ in water intake ($p = 0.22$); the intake EtOH of the Saccharin Group was greater than the Water ($p = 0.002$) and EtOH Group ($p = 0.018$), which did not differ between them ($p = 0.19$). See Figure 3-8(a).

A Bottle (EtOH vs Water) x Stress Group (CTL x SS) X Solution Group (Water x EtOH x Saccharin) ANOVA resulted in a significant interaction between Stress X Solution on the percentage intake of EtOH ($p = 0.004$). For the EtOH and Saccharin Group, rats did not differ (all $p > 0.11$). For the Water Group, SS rats had a higher percentage intake of EtOH than did CTL rats ($p = 0.022$). Among CTL rats, the Water Group had lower percentage intake of EtOH than the other two groups (both $p < 0.001$), which did not differ between them ($p = 0.82$). Among SS rats, the Saccharin Group had greater percentage intake of EtOH than the Water Group ($p = 0.004$; other comparisons $p > 0.11$). See Figure 3-8(b).

### 3.2.7. 24 hours of EtOH Intake (PND 76)

A Bottle (EtOH vs Water) X Stress Group (CTL x SS) X Solution Group (Water x EtOH x Saccharin) ANOVA indicated that rats drank more EtOH than water ($F_{1,44} = 106.54, p < 0.0001$), and that CTL rats drank more than did SS rats ($F_{1,44} = 7.09, p = 0.011$) (all other main effects and interactions, $p > 0.05$; three-way interaction $p = 0.07$), see Figure 3-8(c). The Stress Group X Solution Group interaction on the percentage intake of EtOH was significant ($p = 0.038$), with a lower preference for EtOH in CTL than SS ($p = 0.007$) only among the Water
Group. In a separate analysis of the Stress Group, Solution groups did not differ within CTL rats ($p = 0.08$) or within SS rats ($p = 0.07$), see Figure 3-8(d).

**Figure 3-2.** Chronic intermittent drinking. Mean (± S.E.M.) of drinking across 3 weeks for CTL (control) and SS (adolescent social instability stress) male rats drinking (a) only Water or (b) water and 10% sweetened Ethanol (EtOH). Average intake for each bottle was calculated as grams/kg of body weight for each week (3 exposures). (c) The intake of grams of EtOH ingested was also calculated and averaged for each week.
EXPERIMENT 2: INTAKE FROM TWO BOTTLES

(a) WATER and WATER GROUP

(b) SACCHARIN and WATER GROUP

(c) EtOH and WATER GROUP

(d) EtOH INTAKE

Figure 3-3. Chronic intermittent drinking. Mean (± S.E.M.) of drinking across 3 weeks for CTL (control) and SS (adolescent social instability stress) male rats drinking (a) only Water, (b) water and 0.1% Saccharin or (c) water and 10% sweetened Ethanol (EtOH). The intake for each bottle was calculated as grams of solution/kg of body weight (BW) and the average weekly consumption (mean of the three days of exposure) for each week is shown. (d) The intake of grams of EtOH ingested was also calculated and averaged for each week. * indicates main effect of Stress Group.
Figure 3-4. Elevated Plus Maze. Mean (± S.E.M.) of time spent in the open arm, closed arm, centre, number of entries in closed arm, head dips and rears in the Elevated Plus Maze for CTL (control) and SS (adolescent social instability stress) male rats drinking Water, Saccharin or sweetened Ethanol (EtOH). * indicates main effect of Stress Group. The pattern of means suggested that the differences between SS and CTL rats were driven by rats in the EtOH group, and thus the lack of a significant interaction between Stress Group and Solution Group may be because of insufficient statistical power (Type II) error. The p values along the x-axis indicate the results of post hoc t-tests comparing SS and CTL rats within each Solution Group that were p < 0.05. Nevertheless, these analyses increase the likelihood of Type I error.
Figure 3-5. Social interaction. Mean (± S.E.M.) of time spent in social interaction for CTL (control) and SS (adolescent social instability stress) male rats for CTL (control) and SS (adolescent social instability stress) male rats drinking Water, Saccharin or sweetened Ethanol (EtOH). # indicates significant differences among the Solution Group.

Figure 3-6. Percentage of time spent freezing. Mean (± S.E.M.) of percent freezing time during the fear conditioning test for CTL (control) and SS (adolescent social instability stress) male rats drinking Water, Saccharin or sweetened Ethanol (EtOH).
Figure 3-7. Corticosterone release at different time points. Mean (± S.E.M.) of plasma corticosterone concentration measured at different time points during the fear conditioning test for CTL (control) and SS (adolescent social instability stress) male rats drinking Water, Saccharin or sweetened Ethanol (EtOH). * indicates main effect of Stress Group, # indicates significant differences among Solution Groups.
Figure 3-8. Ethanol intake after withdrawal. Mean (± S.E.M.) of water and EtOH intake (left) and percent intake (right) during (a,b) 1 hour and (c,d) 24 hours after 9 days of withdrawal for CTL (control) and SS (adolescent social instability stress) male rats drinking Water, Saccharin or sweetened Ethanol (EtOH). * indicates a significant difference between Stress Groups either as a main effect or as comparison within subgroups. Matched numbers indicate significant comparisons within Stress Group among Solution Groups. For example, in the left-side panel, superscripts 1 and 2 indicate a higher intake of Water in CTL rats from the Water Group than in CTL rats from the other two solution groups. In the right-side panel, superscripts 1 and 2 indicate a lower percent intake of EtOH in CTL rats from the Water Group than in CTL rats from the other two solution groups.
4. Discussion

We did not find support for our primary hypothesis that social instability stress (SS) in adolescence would increase the propensity to escalate ethanol drinking and/or increase the negative effects of ethanol drinking in adulthood. In experiment 1, SS and CTL rats did not differ in sweetened ethanol intake during its chronic intermittent access, and in experiment 2, SS rats had a reduced intake compared with CTL rats. Nevertheless, three weeks of ethanol drinking did reduce the expression of proteins involved in synaptic plasticity in the prefrontal cortex and dorsal hippocampus as well as reduced concentrations of corticosterone at baseline and at 30 min after fear recall after a week’s absence of sweetened ethanol. On other measures, the effects of chronic intermittent drinking were moderated by stress history: the differences between SS and CTL rats in anxiety-like behaviour were driven largely by the differences within the ethanol group, and the reduction in social interaction in the ethanol group relative to the water and saccharin groups was driven by the effects in CTL rats and were less evident in SS rats. In addition, there was no evidence that ethanol drinking in late adolescence increased the propensity to drink when provided access to sweetened ethanol after nine days of its absence; when intake was measured after 24 hours of access, the percentage intake of ethanol did not differ between those with previous access and those for which it was their first access. Among those drinking sweetened ethanol for the first time (i.e., the Water group), however, SS rats had a greater percent intake of ethanol than did CTL rats at both after 1 hour and 24 hours of access. Thus, SS produced an effect on ethanol intake, increasing its intake in adulthood, and having no effect, or perhaps decreasing its intake, during the late adolescent period. Our finding that SS rats as adults had an increased ethanol intake relative to CTL rats is consistent with other reports using other
stressors in adolescence and tested as adults (Butler et al., 2016; Lesscher et al., 2015; Skelly et al., 2015).

Effectiveness of the chronic intermittent access (IA2BC) manipulation

The three weeks of ethanol drinking manipulation was effective, based on the steady escalation of intake over time. Rats from both CTL and SS groups increased their intake of ethanol over the course of the three weeks of intermittent access, starting at ~5 and reaching ~7 g/kg BW of ethanol intake by the end of the access period. The SS group in experiment 2, however, had a lower consumption of ethanol, starting at ~3 and ending at ~5 g/kg BW of ethanol. Most studies using the IA2BC model, however, expose rats for a longer period of time (6-8 weeks) than used here, typically encompassing pre-puberty into adulthood, and use a higher concentration of ethanol (20%) (reviewed in Carnicella et al., 2014). Most rats under the IA2BC model have a consumption greater than 5 g/kg BW of ethanol on the last week of access, except for the studies that use Sardinian alcohol-preferring rats, that have more than 9 g/kg intake (Sabino, Kwak, Rice, & Cottone, 2013). Thus, the intake in our manipulation is consistent with the intake observed in other studies. A limitation of our studies is that we have no measure of blood alcohol content.

Lack of a reproducible difference in ethanol intake during chronic intermittent access between SS and CTL rats

In experiment 1, SS and CTL rats drank similar amounts of water and ethanol (around 60 g of solution per kg of body weight) during the first week of chronic intermittent drinking, and both groups increased ethanol consumption while decreasing water intake across the access period. In experiment 2, whereas CTL rats had a similar intake pattern to those in experiment 1, SS rats appeared to prefer water rather than sweetened ethanol during the first week of access in...
experiment 2. Both SS and CTL rats progressively increased ethanol consumption over the 3 weeks, but SS rats did not reach the ethanol intake of CTL rats by the end of three weeks of access. One possible explanation for this difference in intake between the two experiments may be cohort variability in susceptibility to the SS procedure. Nevertheless, some of the effects of SS in experiment 2 were consistent with previous research from our lab. For example, SS rats spent less time in social interaction than did CTL rats, which was found in three previous studies (Green et al., 2013; Hodges et al., 2017, 2018). On the other hand, there was no effect of stress on time spent in the open arm of the EPM, in contrast to the reduction previously reported (McCormick et al., 2008). Thus, individual differences in the susceptibility to SS (Hodges et al., 2018) may not have been consistently represented in the different cohorts. Regardless, combined, the evidence suggests any effect of SS on propensity to consume sweetened ethanol when provided with chronic intermittent access in late adolescence is likely very small and in the opposite direction to that predicted. The reduced intake in the present study may be that SS rats are more sensitive to the reduced palatability of sweetened ethanol when provided with continuous access; a pilot study indicated that when provided 24 hr access to saccharin only, SS and CTL rats did not differ, and showed a 90% preference of saccharin to water. Under conditions of shorter access and when competing for access, however, SS rats consumed more unsweetened 10% ethanol than CTL rats and did not differ in intake of 1% sucrose (Marcolin et al., 2019). Thus, the direction of differences between SS and CTL in ethanol intake may depend on the duration of access.

Effects of SS and of chronic intermittent ethanol on protein expression

Dopamine receptors: We hypothesized that adolescent SS may alter dopaminergic function based on our previous studies indicating effects of SS on motivation and reward to
psychostimulants (Mathews, Mills, et al., 2008; Mathews, Waters, & McCormick, 2009). We also predicted that ethanol exposure would alter dopamine receptors based on investigations from other groups that found changes in dopamine receptor expression after ethanol exposure. For example, one study found that repeated ethanol injections during adolescence, but not during adulthood, reduced D2 expression in both nucleus accumbens and prefrontal cortex (Pascual et al., 2009). There was no effect of adolescent stress at either PND 46 or PND 70, nor an effect at PND 70 of ethanol drinking on D1 and D2 receptor expression in the nucleus accumbens or prefrontal cortex. The lack of a difference may also reflect the crude dissections of structures that may have obscured differences that existed within subregions or within cell types of the nucleus accumbens and prefrontal cortex. Further, dopamine receptor expression is not the only adaptation that can alter dopaminergic signalling; alterations in available pools of dopamine, availability of rate limiting enzymes, or response thresholds could also be involved in motivational aspects of drugs of abuse (Nutt, Lingford-Hughes, Erritzoe, & Stokes, 2015). We cannot rule out the possibility that had the drinking of ethanol commenced at an earlier age, or involved a longer period of time, or had we investigated other brain regions, a greater effect on dopamine receptors expression may have been observed.

**Synaptic plasticity proteins:** At PND 46, there was no difference in expression of PSD-95, αCaMKII and βCaMKII between SS and CTL rats in the prefrontal cortex. At PND 70, SS rats from the water group had decreased PSD-95 and αCaMKII expression compared to CTLs. The differential protein expression between CTL and SS rats on PND 70 but not PND 46 indicates a delayed effect of stress, which has been found in other studies from our lab. For example, adolescent SS rats do not differ in spatial location long-term memory from adolescent CTL rats, but when tested as adults, SS rats show reduced memory performance relative to CTL
rats (Green & McCormick, 2013a; McCormick et al., 2010, 2012). Delayed SS effects are not found for all measures, and some effects are present during adolescence and absent in adulthood, such as an increased corticosterone release after forced swim test (Mathews, Wilton, et al., 2008), increased locomotor sensitization to amphetamine (Mathews, Mills, et al., 2008), and increased ethanol intake in short test periods (Marcolin et al., 2019) in SS rats relative to controls. We have reported reduced synaptophysin (another synaptic plasticity protein) expression in the medial amygdala and lateral septum and increased CaMKII in the lateral septum in SS rats compared to CTL rats at PND 46 (Hodges, Louth, Bailey, & McCormick, 2019), increased CaMKII and had no effect on synaptophysin expression in the hippocampus at PND 70 in SS rats than in CTL rats (McCormick et al., 2012). In the current study, however, we found no effect of adolescent SS on CaMKII expression in the hippocampus. Thus, the extent to which the differences observed in synaptic proteins are reliable will require more investigation.

Ethanol drinking reduced expression of PSD-95, αCaMKII and βCaMKII in the dorsal hippocampus of PND 70 rats. Ethanol interacted with stress history in the prefrontal cortex, such that the expression of PSD-95 and αCaMKII in CTL rats that drank ethanol was reduced to that of SS rats, which did not differ across solution groups. A reduction in PSD-95 expression could indicate a deficit in assembling clusters of glutamatergic receptors in the post-synaptic membrane, making it difficult to respond to glutamatergic stimuli coming from other regions (Chen et al., 2011; Keith & El-Husseini, 2008). This reduction might be especially problematic in the prefrontal cortex because it is a key brain structure in decision making and behavioural inhibition (Walton, Bannerman, & Rushworth, 2002). A reduction in αCaMKII in the hippocampus could result in memory impairments, since this kinase has a critical role in learning and memory (Shonesy, Jalan-Sakrikar, Cavener, & Colbran, 2014). As mentioned in the previous
paragraph, we have found memory impairments in hippocampal-dependent memory tasks in adult SS rats. We did not find, however, an effect of stress in fear recall. We also cannot rule out the possibility that the changes in synaptic plasticity markers in the ethanol group might be driven by the saccharin in the solution, because we did not have a saccharin drink group in Experiment 1.

*Effects of chronic intermittent ethanol drinking in experiment 2 on behavioural and corticosterone measures*

Although the differences between SS and CTL rats in the effects of chronic intermittent ethanol drinking on protein expression were modest, effects of ethanol drinking also were moderated by stress history for other measures.

*Anxiety-like behaviour:* Based on the means (see Figure 3), the differences in anxiety-like behaviour between SS and CTL rats appeared to be driven by those that had consumed ethanol during the three weeks of access group. Ethanol consumption in SS rats enhanced the motivation to explore (as measured by number of head dips) and reduced anxiety (as measured by increased time spent in the open arms and reduced time spent in the closed arms) in the EPM test. These effects may reflect Type I error, given that *post-hoc* analysis was conducted within solution groups despite a statistically significant interaction (see Figure 3 legend). There was no difference between SS and CTL rats in the water-only group, which is in contrast to our report of increased anxiety in adulthood after SS in adolescence (McCormick et al., 2008). Our results in SS rats after ethanol exposure are consistent with the finding that ethanol exposure during adolescence (PND 28-42) decreased anxiety when tested in adulthood (Pandey, Sakharkar, Tang, & Zhang, 2015), although others reported it to increase anxiety (Gass et al., 2014). Further, studies that correlated baseline anxiety with later ethanol consumption reported that rats with
higher anxiety levels drank less ethanol compared to rats with low anxiety levels (Acevedo et al., 2016; Acevedo, Nizhnikov, Molina, & Pautassi, 2014). Why an effect of ethanol intake on anxiety was observed only in SS rats remains to be determined, although that SS rats had an overall lower intake than did CTL rats is a likely factor.

**Social interaction:** The reduction in time spent in social interaction among SS rats compared to CTL rats in the Water group failed to meet statistical significance (p < 0.07), and this result contrasts to previous reports from our lab (Green et al., 2013; Hodges et al., 2017, 2018), among rats in the ethanol group, CTL rats interacted the same amount as SS rats, indicating a possible social deficit caused by ethanol. Studies have shown that repeated injection of 1 mg/kg ethanol during adolescence (PND 27-33) reduced social interactions in adulthood (Varlinskaya & Spear, 2007). Our findings suggest that rats will administer sufficient amounts of ethanol under conditions of two-bottle-choice access to produce reductions of social interactions as adults in CTL rats, whereas an effect of ethanol in SS rats may be limited by a floor effect. Further, our results suggest that late adolescence (> PND 46) remains a vulnerable period for ethanol’s effects on social interaction.

**Contextual fear conditioning:** We previously reported that SS rats had a reduced recall of either cue or context than did CTL rats after cued fear conditioning in the absence of any ethanol exposure (Morrissey, Mathews, & McCormick, 2011), as well as reduced performance on other hippocampal-dependent measures (spatial memory) (Green & McCormick, 2013b; McCormick et al., 2010, 2012). Thus, we had predicted that SS would show reduced context recall after contextual fear conditioning, and that ethanol exposure might exacerbate this effect in SS rats. We did not predict this effect of ethanol for CTL rats based on the report that adolescent exposure (PND 26-66) to 20% ethanol in IA2BC protocol did not alter fear responses (Pajser,
Breen, Fisher, & Pickens, 2018). Our prediction was not confirmed; we found no difference in recall between SS and CTL rats nor an effect of ethanol exposure. This discrepancy in an effect of SS on fear recall may be because of methodological differences in the fear protocol (context conditioning rather than cue conditioning).

**Corticosterone concentrations:** Rats that had chronic intermittent access to ethanol had a lower concentration of corticosterone at baseline and 30 minutes after fear recall compared to rats exposed to the other solutions. Acutely, ethanol typically increases plasma corticosterone concentration by activating the hypothalamic-pituitary-adrenal (HPA) axis, but chronic ethanol exposure can blunt HPA reactivity (reviewed in Rivier, 2014). For example, rats exposed to ethanol vapors during adolescence (PND 28-42) exhibited reduced mRNA expression of proteins involved with HPA activity and reduced neuronal activity in the paraventricular nucleus of the hypothalamus (PVN) after ethanol challenge in adulthood (Allen, Rivier, & Lee, 2011; Logrip et al., 2013). Based on these studies, one possible explanation for the lower corticosterone here is that ethanol chronic exposure and/or its subsequent removal from access dysregulated the HPA axis (Allen, Lee, Koob, & Rivier, 2011). Reduced baseline corticosterone or blunted corticosterone release can be an indication of dysregulated HPA activity (Lupien, McEwen, Gunnar, & Heim, 2009). The withdrawal from ethanol after days or weeks of exposure can reduce secretion of adrenocorticotropic hormone (ACTH, which stimulates the release of corticosterone), corticosterone and mRNA expression of secretagogues of ACTH (corticotropin releasing factor and vasopressin) in the PVN where the HPA response is initiated (reviewed in Rivier, 2014). The withdrawal effect on HPA dysregulation could also explain the different corticosterone concentrations in rats from the saccharin group that was evident at some time.
points, and withdrawal from saccharin may also be part of the effect in the sweetened ethanol group.

SS rats had lower corticosterone concentrations than CTL rats at almost all time points tested. Stressors tend to increase corticosterone release: six weeks of social isolation (PND 28-70) increased corticosterone release after forced swim test (PND 85) and prevented suppression of release after dexamethasone challenge (Butler, Ariwodola, & Weiner, 2014). Also, previous exposure to ethanol during adolescence (PND 37-44, i.g.) and exposure to restraint stress in adulthood (PND 74) independently increased corticosterone release (Torcaso, Asimes, Meagher, & Pak, 2017). Regarding the social instability stress used in this study, we typically do not find differences between SS and CTL rats as adults in corticosterone release when rats are exposed to a second stressor: 30 minutes of restraint stress (McCormick et al., 2005), 15 minutes of forced swim (Mathews, Wilton, et al., 2008), and 15 minutes of EPM (McCormick et al., 2008). This same lack of a difference in corticosterone concentration between SS and CTL rats was found in the present study for the water group. Nevertheless, social instability stress in adolescence may render rats more sensitive to the dampening effect of sweetened ethanol exposure and/or its withdrawal on corticosterone release, as described earlier. Our data suggest these effects do not involve changes in GR; there was no effect of stress history or of chronic intermittent ethanol drinking on GR expression at PND 70.

**History of ethanol drinking and subsequent exposure to ethanol**

After nine days of ethanol’s absence, SS rats drank less ethanol than did CTL rats after 1- and 24-hours access, and neither group drank more than did rats that drank saccharin during the three weeks of intermittent access. Thus, a history of ethanol intake during late adolescence did not increase the propensity to drink ethanol in adulthood. Although this result was based on
intake in one day only, the result is in contrast to evidence for an increased risk of ethanol consumption in adulthood when drinking commences at an earlier age in humans (Bonomo et al., 2004; Odgers et al., 2008). The pre-clinical literature has mixed results: some studies have shown that the exposure to ethanol during adolescence by injection, gavage, vapor, or voluntary drinking increases ethanol intake later in life (Alaux-Cantin et al., 2013; Amodeo, Kneiber, Wills, & Ehlers, 2017; Fernandez et al., 2016; Gass et al., 2014; Hargreaves, Monds, Gunasekaran, Dawson, & McGregor, 2009; Labots et al., 2018; Maldonado-Devincci et al., 2010; Pandey et al., 2015; Pascual et al., 2009; Rodd-Henricks et al., 2002; Yoshimoto et al., 2002), whereas other studies do not find such an effect (Amodeo et al., 2018; Füllgrabe et al., 2007; Gilpin et al., 2012; Juárez & Vázquez-Cortés, 2003; Milivojevic & Covault, 2013; Nentwig, Margaret Starr, Judson Chandler, & Glover, 2019; Palm & Nylander, 2014; Schramm-Sapyta et al., 2014; Siegmund et al., 2005; Slawecki & Betancourt, 2002; Varlinskaya, Kim, & Spear, 2017; Vendruscolo et al., 2010; Vetter et al., 2007). It appears that the method itself does not influence the likelihood of adolescent rats to increase ethanol intake in adulthood, but it might be a combination of multiple factors: method (voluntary x involuntary), age of initial intake (prepubertal, postpubertal, early adulthood), frequency of intake (continuous x intermittent), sex, strain. More studies are required to understand the influence of such moderators.

Ethanol-naïve SS rats drank more ethanol as adults than did ethanol-naïve CTL rats after both 1- and 24 hour-access, indicating that the history of adolescent SS may increase propensity to drink ethanol in adulthood. In a previous study, adolescent SS increased ethanol intake during short periods of ethanol access (5 min, 30 min or 1 hour) in mid-late adolescence, but did not alter intake during adulthood (Marcolin et al., 2019). In the current study, SS rats were drinking
less ethanol during the intermittent access period from mid-adolescence into adulthood, which suggests a longer test period may be required to observe differences between SS and CTL rats tested as adults, whereas a higher intake in SS rats in adolescence may only be evident when access is limited.

Rats that drank 0.1% saccharin during the intermittent access period drank more ethanol than did rats in the water group (but not those that had previously consumed sweetened ethanol), indicating that previous experience with saccharin may have reduced the neophobia associated with the ethanol solution. Saccharin is very palatable to rats, and there are studies showing that rats self-administer more saccharin than ethanol (Russo, Funk, Loughlin, Coen, & Lê, 2018). Nevertheless, our results do not indicate that saccharin altered the behavioural measurements to the same extent that ethanol did, and, with the exception of corticosterone levels, the results for saccharin rats were very similar to those for water rats.

Conclusions

In summary, the effects of chronic intermittent ethanol drinking were modest and were moderated by adolescent SS for several measures. Exposure to ethanol in late adolescence/early adulthood attenuated differences between CTL and SS rats on levels of αCaMKII and PSD-95 in the prefrontal cortex and in the social interaction test, which were evident only in water exposed rats. SS rats had decreased anxiety-like behaviour in the Elevated Plus Maze, with the effect driven largely by the difference between SS and CTL rats in the ethanol exposure group. Exposure to ethanol had the same effect in SS and CTL rats in reducing αCaMKII, βCaMKII, and PSD-95 in the dorsal hippocampus, and reducing baseline and fear recall recovery concentrations of corticosterone relative to those exposed only to water. SS rats typically had
lower corticosterone concentrations at baseline and fear recall and recovery time points than did CTL rats. The interaction between stress and chronic intermittent ethanol drinking was also found when testing the propensity to drink ethanol later in adulthood: SS increased ethanol intake only in ethanol-naïve rats, whereas previous exposure to ethanol was not able to increase later intake. A next step will be to repeat the experiment in female rats, because the results obtained here are likely sex-specific. We have found sex-specificity in the effects of SS for several measures, including corticosterone concentrations during the SS procedure (McCormick et al., 2007), locomotor sensitization to nicotine (McCormick et al., 2004, 2005) and amphetamine (Mathews, Mills, et al., 2008), and on measures of anxiety (McCormick et al., 2008). Further, male and female rats are known to differ in ethanol intake, with females typically drinking more than males (Amodeo et al., 2018; Doremus et al., 2005; Westbrook et al., 2018), and to differ in sensitivity to ethanol-induced effects such as social interactions (Kim, Varlinskaya, Dannenhoffer, & Spear, 2018), drinking under social conditions (Varlinskaya et al., 2015a), aversity to ethanol during conditioning taste aversion (CTA) (Vetter-O’Hagen et al., 2009), sensitivity to restraint stress (Wille-Bille, Ferreyra, et al., 2017) and baseline corticosterone concentration (Przybycien-Szymanska, Rao, & Pak, 2010). The present results in male rats evidence the complex interaction between a history of stress exposures and of ethanol drinking during development on adult function, and further studies are necessary to unveil the neural mechanisms underlying the complexities.
Chapter 4: General Discussion

The main goal of this thesis was to investigate the effects of adolescent social instability stress (SS) on immediate and long-lasting changes on reward-related behaviours in male rats using voluntary alcohol intake paradigms. Another goal was to investigate the influence of social context on the propensity to drink alcohol, as well as the influence of these factors on sucrose intake. There has been an increase in research over the last two decades about the adolescent period as a vulnerable period for the consequences of stressors and drugs of abuse (Burke & Miczek, 2014), and the present work intended to investigate this area using a so far underexplored model of adolescent social stress.

Effect of SS on alcohol intake

The first hypothesis that adolescent SS would affect the propensity to drink alcohol soon after the stress was confirmed in this thesis. In chapter 2, I demonstrated that adolescent SS from PND 30-45 increased alcohol intake soon after the stress ended. My study was the first to investigate the effect of this particular model of adolescence social instability stress on alcohol intake in adolescent rats. Even so, my results can be compared to other studies using different models of adolescent social stress. For example, adolescent male rats socially isolated for three weeks starting on PND 22 had higher alcohol consumption compared to non-stressed rats (Van Waes et al., 2011). In contrast, adolescent male rats socially isolated for 12 days starting on PND 26 showed no difference in alcohol consumption (Doremus et al., 2005). The difference between the studies could be related to the length of the stressor, only encompassing prepuberty in the Doremus study, and ending after puberty in my and Van Waes’ study. It has been shown that
exposure to social stressors beginning in prepuberty and extending into puberty have different consequences on alcohol intake than stressors that start only after puberty (Spear, 2015). Thus perhaps stressors that are restricted to prepuberty could also have a different impact on alcohol intake than pre and post puberty stressors.

Based on previous studies with amphetamine and nicotine, in which SS increased locomotor sensitization to these drugs in adulthood (Mathews, Mills, et al., 2008; McCormick & Ibrahim, 2007; McCormick et al., 2004, 2005), we had predicted that SS would also increase alcohol consumption in adulthood. In chapter 2, we did not find a strong influence of SS on alcohol intake among adult rats; adult SS rats did not differ from CTL rats in the first (Figure 2-2c) and third experiments (Figure 2-6a), whereas all SS rats had higher alcohol intake irrespective of age in the second experiment (Figure 2-4c). In contrast, however, other studies using different models of adolescent social stress have found that exposure to adolescent stress increased alcohol intake in adulthood. For example, adolescent male rats socially isolated for 3 weeks starting on PND 21 (Lesscher et al., 2015), or for 6 (Butler, Carter, et al., 2014; Skelly et al., 2015) or 13 weeks starting on PND 28 (Chappell et al., 2013) had higher alcohol intake in adulthood compared to non-stressed rats. During social isolation, the rats are kept alone in smaller cages throughout the weeks, which is considered a strong model of social deprivation. During SS, in contrast, the rats are isolated for 1 hour and return to a different cage partner every day from PND 30-45, which is thought to disrupt the social repertoire of the rats without socially depriving them, and therefore, being considered a mild stress model compared to social isolation. Thus, the methodological differences between social isolation (SI) and SS in terms of age of onset of the stressor (early adolescence in SI, mid adolescence in SS), duration of the stressor
(many weeks in SI, 16 days in SS) and the intensity of the stressor (SI strong, SS mild) could perhaps explain the lack of increased alcohol intake among adult SS rats.

As just mentioned, in chapter 2, the SS procedure increased alcohol intake during adolescence, but this effect did not persist into adulthood. On the other hand, in chapter 3, the same adolescent SS model reduced alcohol intake from late adolescence until adulthood. This difference in outcomes between the two studies could be explained by several factors. First, the different methodology of access to alcohol, short periods in chapter 2 (5, 30 or 60 min) compared to long periods in chapter 3 (24 hours 3x/week). Given that short access resulted in higher consumption by SS rats, whereas longer access resulted in lower consumption by SS rats, perhaps the amount of time available for access to alcohol could influence the consumption patterns in response to stress. If that is the case, we would also expect to find reduced alcohol intake in SS rats whenever they have longer access to alcohol. But we found the opposite result in chapter 3: when adult SS rats that were naïve to alcohol (water group) had access to alcohol for the first time, they drank more alcohol in 24 hours than CTL rats (Figure 3-8b). This result suggest that the availability of alcohol alone might not be the only factor influencing the sensitivity to SS effects. Perhaps the history of alcohol drinking associated with prolonged intake period reduces the ability of SS to increase alcohol intake.

A second possible explanation for the opposite SS effect in alcohol intake between the chapters is the place in which the rats had access to alcohol. In chapter 2, rats were drinking alcohol in experimental boxes without any physical contact (separated by a mesh wall), whereas in chapter 3 the cage mates were drinking in their home cage while in physical contact. We have previously demonstrated that the SS procedure affects the social repertoire of the rats. For example, during social interaction, a behavioural test in which rats investigate an unfamiliar
stimulus rat, SS rats spend less time investigating the novel rat than CTL rats when tested as both adolescent and adult (Green et al., 2013; Hodges et al., 2017, 2018) (also confirmed in Figure 3-5). However, during social approach, a behavioural test in which the stimulus rat is confined behind a meshed cage, SS rats spend as much time as CTL rats investigating the novel rat secured behind a mesh (Green et al., 2013; Hodges et al., 2017). So perhaps the reduction in alcohol intake in SS rats (or absence of effect) in chapter 3 was induced by the awkwardness of the SS rats in having to drink while physically interacting with their cage partners, which was not an issue in chapter 2. It would be interesting to investigate whether socially isolated rats would also show a decrease in alcohol consumption if they were drinking in pairs or groups of more rats.

A third possibility for the opposite SS effect in alcohol intake between the chapters is the difference in the alcohol solutions used. In chapter 2, rats had access to unsweetened 10% alcohol, whereas in chapter 3, rats had access to 10% alcohol sweetened with 0.1% saccharin. The presence of sweeteners could alter the motivation to drink alcohol compared to a non-sweetened solution. Stressors usually increase the intake of sweet substances, and we have shown that SS increases intake of sweet condensed milk in adulthood (Cumming et al., 2014). Therefore, the effect of SS on the consumption of sweetened alcohol should have been greater in chapter 3 than in chapter 2. Contrary to this prediction, SS rats in chapter 3 drank equal or lower amount of sweetened alcohol than CTL rats during the 3 weeks of intermittent access and after 9 days of abstinence, only increasing the intake among alcohol-naïve rats. During the relapse test, among the alcohol group, CTL rats had higher preference to drink alcohol than SS rats within one hour, but that difference disappeared in 24 hours. It seems that the effects of SS on alcohol drinking are dependent on the age of testing. For adolescent rats, the critical factor seems to be
the length of access: SS increases alcohol intake during short periods of access (Figures 2-2c, 2-4c, 2-6a), but decreases or does not change intake during prolonged period of access (Figures 3-2b,c, 3-3c,d). For adult rats, the critical factor seems to be the history of alcohol drinking, not the length of access: SS rats naïve to alcohol drink more during short or longer access than CTL rats (Figure 3-8a,c), whereas SS rats with a history of alcohol drinking have lower or similar intake over short and longer access (Figure 3-8a,c). These results suggest a complex interaction between age, history of stress exposure and of alcohol drinking in the modulation of the reward system to influence motivation to drink.

One limitation in chapter 3 was the lack of measurement of alcohol drinking within 30 or 60 min from the first day of intermittent access. This way we could have better compared the short period of access of a sweetened solution with the 24-hour access at any given age to the consumption in chapter 2. We also don’t know whether the high alcohol intake of EtOH-naïve SS rats would have continued to be high over time, or started to decrease and match the level of CTL rats. Further tests are required to answer this question. It would also be interesting to identify whether these effects of alcohol intake are similar using an unsweetened alcohol solution in chapter 3.

**Effect of SS on sucrose intake**

The hypothesis that adolescent social stress would also affect sucrose intake was partially confirmed in chapter 2. SS rats, irrespective of age, consumed more 1% sucrose than CTL rats, but only during a competition setting (Figure 2-7a). This result is consistent with a previous study from our lab evaluating adolescent SS effect on sweetened condensed milk competition in adulthood (Cumming et al., 2014). The amount of time that the rats spent drinking sucrose on the
5th day of testing in chapter 2 was around 50 seconds, whereas in the test with sweetened condensed milk in Cumming study rats spent around 100 seconds. This difference could be related to the higher palatability of sweetened condensed milk (which contains about 30% sucrose). We also found that the consumption of 1% sucrose was greater for adolescent than for adult rats in all three tests in chapter 2, which is consistent with greater sensitivity for sweet solutions at that age (Friemel et al., 2010). However, not all stressors increase the consumption of sucrose solutions. For example, male rats socially isolated from PND 30-50 did not differ in sucrose intake in adulthood compared to non-stressed rats (Hong et al., 2012). A mixed stressor procedure (isolation and social defeat) from PND 36-48 decreased sucrose consumption soon after the end of the stress procedure in male rats (Harrell et al., 2013). These results indicate that SS effects are not entirely exclusive to drugs of abuse, even though in chapter 2 the amount of time rats spent drinking sucrose was much higher than the time spent drinking alcohol.

**Effect of social context on alcohol intake**

The hypothesis that the social context would influence alcohol drinking was not supported by the results. In chapter 2, rats drinking alone drank the same as rats drinking with peers (familiar or unfamiliar), which is not consistent with reports of more drinking among peers than alone (Varlinskaya et al., 2015a). This difference in outcome might be due to methodological differences between the studies. Our rats were unfamiliar to each other and tested over one session either alone or in the presence of a peer while kept apart by a wire mesh. On the other hand, in the Varlinskaya study rats were tested in groups of 4 to 5 cage mates for 6 drinking sessions alternating between the alone and social condition without any physical separation. Furthermore, our study involved the use of unsweetened 10% alcohol, whereas the
Varlinskaya study used 10% alcohol sweetened with 3% sucrose and 0.125% saccharin. Given all the methodological differences, it is hard to draw a proper comparison and conclusion from these results. More studies involving the social aspect of drinking in rats should give us more answers.

**Effect of SS and alcohol on the brain**

The reason why there are differences in alcohol intake between SS and CTL rats are unknown. One hypothesis is that stress alters the reward system, which then affects propensity to drink. In chapter 3, I failed to find any effect of SS on the reward system; there were no differences in protein expression the day after the stress was over (Table 3-1). The history of ethanol drinking reduced synaptic plasticity markers in the dorsal hippocampus and prefrontal cortex, and this reduction was sometimes further reduced by SS (Table 3-2). But contrary to our predictions, there was no difference in dopamine receptors 1 and 2 in adult rats. These results only partially confirm the hypothesis that adolescent SS would modify the reward system and increase propensity to drink. Perhaps the amount of alcohol consumed by the rats was not sufficient or prolonged enough to induce profound changes in the dopaminergic system and synaptic plasticity in the brain regions analyzed in this study. For example, 28 days of continuous access to alcohol (average intake of 9.7 g/kg BW) decreases CaMKII expression in the nAcc (Zhao et al., 2015) of adult rats. Also, 8 repeated injections of 3 g/kg of alcohol (reaching BAC of 195 mg/dl) decreased expression of D2 receptors in the nAcc, PFC, hippocampus and striatum of adolescent, but not adult rats (Pascual et al., 2009). And even though dopamine receptor expression is not the only adaptation that can alter dopaminergic signalling and motivation (Nutt et al., 2015), the absence of differences in dopamine expression
does not explain the difference in drinking behaviour. We also have evidence from our lab that SS reduced synaptophysin (another synaptic plasticity protein) expression in the medial amygdala and lateral septum and increased CaMKII in the lateral septum at PND 46 (Hodges, Louth, Bailey, & McCormick, 2019), and increased CaMKII but had no effect on synaptophysin expression in the hippocampus at PND 70 (McCormick et al., 2012). Therefore, other brain regions affected by SS such as the amygdala, lateral septum and the bed nucleus of the stria terminalis should be investigated to assess the extent of changes caused by alcohol access.

**Implications**

The peer pressure of alcohol exposure seems to be a factor for human adolescent alcohol intake, and very few studies have been conducted to assess whether peer pressure is present in rats as well. Our study shows no differential alcohol intake among rats drinking alone or with a peer. This result suggest that peer pressure among adolescent rats still needs to be further investigated before translating the finding back to humans.

The increased alcohol intake among stressed rats, particularly during adolescence, is in accordance with other models of social stressors in the literature and raises concern regarding the heightened risk of drug abuse problem among teenagers. On the one hand, this increased propensity to drink among stressed rats does not seem to persist in adulthood, so perhaps this transient effect could be protective in order to not increase the risk of future drug of abuse. On the other hand, the consequences of prolonged alcohol intake is present in adulthood, although the results we found were quite modest in a few brain regions. Both stress and alcohol were able to cause modifications in brain regions that are part of the reward system, and more studies are necessary to investigate the extent of changes caused by stress and alcohol.
**Future perspectives**

In humans, males are more likely to develop alcohol use disorder. However, women drinking excessively may develop more medical problems (reviewed in Erol & Karpyak, 2015). Also, women become intoxicated at a faster rate and become ethanol-dependent more rapidly than do men (Mancinelli, Vitali, & Ceccanti, 2009). In rodents, sex is also an important methodological consideration as evident in several of the studies described earlier. Females have been, and continue to be, under-studied in research, and the results in one sex do not readily translate to the other sex. Many studies point to sex differences in both the appetitive and consummatory responses to ethanol in adolescent and adult rodents, with females typically (though not always) showing greater intake relative to males (Amodeo et al., 2018; Broadwater et al., 2013; Doremus et al., 2005; Lancaster & Spiegel, 1992; Tambour et al., 2008; Varlinskaya et al., 2015b; Westbrook et al., 2018; Wille-Bille, Ferreyra, et al., 2017, but also see Dhafer et al., 2012; Vetter-O’Hagen et al., 2009). There is also evidence that the presence of peers may promote ethanol intake in rodents, and more so in adolescence than in adulthood and more so in male rats than in female rats (Logue et al., 2014; Varlinskaya et al., 2015a). Thus, it will also be important to investigate the effects of SS on drinking in females.

**Conclusion**

Adolescence is a sensitive period in which the effects of stress and alcohol can have long-lasting impacts (Crews et al., 2019). My findings indicate that using a model of mild social stressor can have great impact on adolescent rats, but moderate effects in adult rats. The behavioural changes caused by stress can be enhanced by history of alcohol drinking later in life,
but that does not necessarily causes an increase in the propensity to drink during adulthood. Adolescent stressed rats drink more alcohol than other groups, but they don’t seem to continue drinking more when they reach adulthood. These results indicate that the effects of social instability stress might be transient in regards to propensity to drink, and can be the basis for alterations caused by both alcohol and stress.
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