3α-hydroxy-5α-pregnan-20-one:

Alcohol Consumption and Anxiety in $GABA_A$ α2 knock-in C57Bl/6 Mice

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Abstract

Allopregnanolone has demonstrated anxiolytic and anticonvulsant properties via a GABAergic mechanism of action similar to that of benzodiazepines. Intermittent exposure to ethanol has produced high levels of alcohol consumption in the C57Bl/6 mice strain (Hwa et al., 2011). Withdrawal from alcohol produces anxiogenic effects in many species. A possible synergist action for ethanol and the endogenous neurosteroid allopregnanolone may exist at the $GABA_A$ receptor. Male and female mutant C57Bl/6 $GABA_A$ α2 knock-in allopregnanolone insensitive mutants were subjected to the intermittent access protocol for eight weeks and then analyzed for severity of alcohol withdrawal using the Handling Induced Convulsion (HIC) score (Goldstein, 1972). Animals were tested for anxiety-like behaviors during ethanol withdrawal in the open field and social preference tests after injections of 5.0 mg/kg and 10.0 mg/kg allopregnanolone and 0.56 mg/kg midazolam. There were no main effects for increased anxiolytic behaviors across genotypes post allopregnanolone and midazolam injection; however main effects for sex and ethanol withdrawal were discovered. Male animals and ethanol naïve animals displayed less anxiety-like behaviors in the social preference and open field tests, regardless of drug treatment. This study suggests that ethanol and allopregnanolone behavioral effects are mediated through a similar neural pathway that is likely to be related to action at the GABA-A receptor.

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Alcoholism is a global phenomenon, with the highest levels of alcohol consumption reported from developed countries in the Northern Hemisphere (WHO, 2011). An average of 6.13 liters of alcoholic beverages was consumed per capita worldwide in 2005 by every person aged 15 years or older according to the World Health Organization's Global Status Report on Alcohol and Health (WHO, 2011). Epidemiological problems with alcohol consumption also include high incidences of heavy episodic drinking which have been found to influence the rates of crime and violent behavior worldwide (WHO, 2011). Alcoholism has also been linked to anxiety disorders; people with pre-existing generalized anxiety disorder have a higher risk for developing alcohol dependence (Kessler et al., 1997; Liang & Chikritzhs, 2011). There are also a number of differences related to alcohol consumption in men and women: while men tend to consume more alcohol than women (Dawson & Archer, 1993; Vengeliene, Bilbao, Molander, & Spanagel, 2008), it is more likely for women to become alcoholics if they have had a first-degree relative with depression (Weissman & Klerman, 1977). The prevalence of alcohol consumption globally and its related negative effects on personal health and society at large is cause for the further study and understanding of the neural mechanisms of alcohol.

Previously, alcohol has been considered with an unspecific site and mechanism of action (Crews, Morrow, Criswell, & Breese, 1996), however studies have now shown that alcohol does have specific neural sites of action at opioid, nicotinic, the glutamate *N*-methyl-D-aspartate NMDA, glycine, 5-HT_3, dopamine, corticotrophin-releasing factor (CRF), and $GABA_A$ receptors (Vengeliene et al., 2008). The primary GABAergic target for alcohol is $GABA_A$ receptors

containing alpha subunits (Vengeliene et al., 2008). There is evidence that aspects of alcohol dependence and withdrawal are influenced by alterations in $GABA_A$ receptor function (Morrow, 1995). The $GABA_A$ receptor has also been linked to anxiolytic effects, where blockade of GABAergic inhibition at the $GABA_A$ receptor regulated anxiety responses in social interaction and conflict tests in rats (Sanders & Shekhar, 1995). An endogenous neurosteroid, allopregnanolone (3-alpha-hydroxy-5-alpha-pregnan-20-one), is a positive allosteric modulator of the $GABA_A$ receptor and has demonstrated protection for seizure susceptibility after acute ethanol withdrawal in rats (Morrow, 1995). Fluctuation in the concentration of allopregnanolone has been shown to effect the expression of $GABA_A$ receptors and allopregnanolone withdrawal has demonstrated increased anxiolytic behavior in rats (Follesa, Biggio, Caria, Gorini, & Biggio, 2004). The link between anxiolytic behavior following withdrawal from acute ethanol administration and the modulatory action of allopregnanolone at the $GABA_A$ receptor suggests a possible synergist action of endogenous neurosteroids and alcohol in modulating anxiety-like behavior. The overall aim of this study is to further analyze the neural interaction of alcohol and allopregnanolone at the $GABA_A$ receptor and how this interaction affects alcohol consumption, withdrawal, and anxiety-like behavior.

Ethanol: Chemical properties and pharmacologic action

The psychoactive component of all alcoholic beverages is ethanol. Ethanol impairs cognitive processing and disrupts motor coordination in addition to anxiolytic, sedative hypnotic, and anticonvulsant effects (Grobin, Matthews, Devaud, & Morrow, 1998). High concentrations produce anesthetic effects and depress the respiratory system (Grobin et al., 1998). Ethanol activates the hypothalamic-pituitary axis and increases plasma levels of certain steroids including corticosterone, testosterone, and progesterone (VanDoren et al., 2000). It was previously

hypothesized that this hydrophilic molecule acts on hydrophobic portions of cells by partitioning the lipid cell membrane and thus altering the cell's physical properties and function, leading to intoxication and anesthesia (Deitrich, Dunwiddie, Harris, & Erwin, 1989). More recent studies have demonstrated the action of ethanol on ligand-gated ion channels in the nervous system. Ion channels function to initiate rapid synaptic activity and propagate action potentials (Crews et al., 1996). Ethanol interacts with these ion channels by entering molecular sites and modifying the intermolecular bonds important for the open-close kinetic action of the channels (Crews et al., 1996). Several classes of neuronal receptors have been found to react directly and indirectly with ethanol, each leading to different alcoholic symptom severity and behavioral changes.

Ethanol has indirect effects at the opioid G-protein coupled receptors. High doses of morphine have been shown to decrease alcohol consumption, and the "opioid compensation hypothesis of alcoholism" postulates that a deficiency in endophinergic activity is compensated for by ethanol intake (Herz, 1997). Endogenous opioids have also a less direct mediating effect of dopaminergic mechanisms on the effects of alcohol; the dependence-producing properties of opiods enhance DA neurotransmission (Herz, 1997). The reinforcing effect of morphine and the aversive effects of the opioid antagonist naloxone can be abolished by antagonism of the D1 receptor (Herz, 1997). The reinforcing role of the dopaminergic mesolimbic reward pathway in alcohol dependence is seen when dopamine neurons in the VTA increase firing rates dose-dependently to doses of ethanol administered intravenously in rats (Gessa, Muntoni, Collu, Vargiu, & Mereu, 1985). Corticotropin releasing factor (CRF) is also implicated in behavioral stress responses to alcohol withdrawal. Antagonism of the G-protein coupled CRF-1 receptor in rats was found to reduce the anxiogenic effect of alcohol withdrawal (Heilig & Koob, 2007).

Acute alcohol administration was also found to upregulate expression of the CRF-1 receptor in the rat hypothalamus (Lee & Rivier, 1997).

Ethanol has a more direct effect at receptor-activated ion channels including the inhibition of subtypes of the NMDA-glutamate receptor and potentiation of subtypes of $GABA_A$ receptor (Crews et al., 1996). Alcohol-induced inhibition of responses to NMDA receptor activation may contribute to the neural and cognitive impairments associated with intoxication (Vengeliene et al., 2008). Ethanol has also been shown to potentiate neuronal nicotinic receptors as well as directly modulating the ligand-gated 5-hydroxytryptamine 3 (5-HT3) receptor (Vengeliene et al., 2008). The excitatory 5-HT3 receptor is often expressed on GABAergic neurons, and activation of 5-HT3 receptors by ethanol may contribute to some of the inhibitory actions of alcohol via the increased release of $GABA_A$ (Lovinger, 1999). The interaction of ethanol with various different receptors in specific areas of the brain has implications for the behavioral and addictive effects of acute alcohol consumption. The interaction of ethanol at the $GABA_A$ receptor is the focus of this study.

The $GABA_A$ Receptor

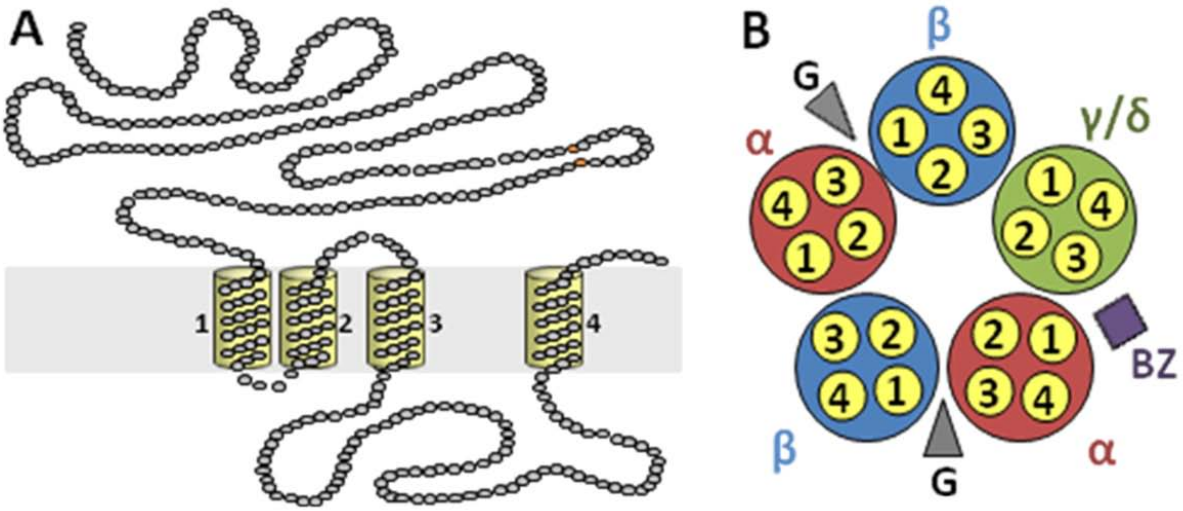
Function

γ-Aminobutyric acid (GABA) is an inhibitory neurotransmitter in the central nervous system (Hayashi, 1958) that acts at approximately one-thirds of all synapses (Sieghart & Sperk, 2002). The $GABA_A$ receptor is a ligand-gated ion channel that controls influx of calcium into the cell (Sieghart & Sperk, 2002). There are two forms of GABAergic inhibition: phasic inhibitory postsynaptic currents due to vesicular release, and tonic conductance activated by low ambient GABA concentrations (Vicini & Ortinski, 2004). Tonic conductance, originally identified in rat cerebellar granule neurons (Kaneda, Farrant, & Cull-Candy, 1995), has also been identified in

dentate gyrus' granule neurons, thalamic neurons, and hippocampal interneurons and pyramidal cells (Vicini & Ortinski, 2004). $GABA_A$ receptors that exhibit tonic conductance of GABAergic inhibition are sensitive to low concentrations of ethanol and neuroactive steroids (Vicini & Ortinski, 2004).

Composition

The mammalian $GABA_A$ receptor is made up of a combination of glycoproteins which span the membrane and combine to form five subunits (Loh & Ball, 2000). Each receptor is comprised of a pentameric arrangement of subunits (Sieghart & Sperk, 2002). There are seven classes of subunit, and each has a variety of different isoforms: six α, four β, three γ, one ε, one π, one δ and one θ, with the most common subunit combination including two α, two β, and one γ (Sieghart & Sperk, 2002). All $GABA_A$ receptor subunits include a large N-terminal extracellular domain, four transmembrane (TM) domains, and a large intracellular loop between TM3 and TM4 (Sieghart & Sperk, 2002). The effect of the different combinations of subunits on behavior has been a focus for $GABA_A$ receptor research. The figure below illustrates the transmembrane domains as well as the five subunit composition of the $GABA_A$ receptor:



Subunit composition and transmembrane domains of the GABAA receptor (K. N Gurba 2010).

Behavior

Genetically modified mouse models have demonstrated that benzodiazepines, barbiturates, alcohol, and anticonvulsants have receptor sites on the $GABA_A$ receptor, and certain $GABA_A$ subunits have an effect on behavior and drug sensitivity (Grobin et al., 1998; Loh & Ball, 2000). The convulsion-inducing chemical picrotoxin has a binding site on the GABAA receptor (Holland, McKeon, Covey, & Ferrendelli, 1990). Benzodiazepine binding at the $GABA_A$ receptor is partially inhibited by convulsant alkyl-substituted gamma-butyrolacetones and gamma-thiobutyrolacetones by displacement of the ligand [^{35}S]t-butylbicyclophosphorothionate ([^{35}S]TBPS) which binds to the picrotoxin receptor (Holland et al., 1990) Injections of the $GABA_A$ receptor agonist muscimol directly into the median raphe nucleus reinstated extinguished alcohol seeking behavior in rats (Vengeliene et al., 2008). The different isoforms of the alpha subunit and their role in determining behaviors associated with the $GABA_A$ receptor such as sedation, sleep induction, myorelaxation, anxiety, seizures, and amnesia have been the focus of multiple mouse knock-out and knock-in studies (Vicini & Ortinski, 2004). A region of 45 amino acids on TM2 and TM3 of the $GABA_A$ receptor subunits α1 and α2 is necessary for the enhancement of $GABA_A$ receptor function by alcohol (Mihic et al., 1997). $GABA_A$ receptors that contain alpha subunits are primary targets for alcohol, as opposed to their counterparts that contain gamma subunits (Vengeliene et al., 2008). Cloning of the alpha subunits in mice demonstrates that the α2 subunit is localized to the synapse along with the alpha-1 and alpha-3 subunits, while α-subunits 4-6 are localized outside of the synapse and are considered “extrasynaptic” (Vicini & Ortinski, 2004). There have been multiple studies investigating the different roles for the $GABA_A$ alpha subunits and their pharmacological responses to ethanol and benzodiazepines.

The extrasynaptic receptors have not demonstrated direct correlations with ethanol action at the $GABA_A$ receptor. The $GABA_A$ receptors containing α4 subunits are predominantly expressed in hippocampal regions in C57Bl/6 mice (Cestari, Liu, Mu, & Burt, 1998). This subunit has an unclear role in ethanol withdrawal symptoms since α4 subunit mRNA and protein level changes after ethanol exposure are also accompanied by changes in other subunits, most notably the δ subunit (Vicini & Ortinski, 2004). The α5 subunit is mainly expressed in the hippocampus, and has demonstrated a role with learning and memory (Vicini & Ortinski, 2004). A study with α5 knock-in mice rendered animals insensitive to the diazepam, but the mutation had no effect on the anxiolytic, sedative and anticonvulsant effects of the benzodiazepine, suggesting that this subunit does not have a role in mediating these behavioral effects (Crestani, Assandri, Tauber, Martin, & Rudolph, 2002). The α6 subunit, expressed in the cerebellum, is a mediator for spillover tonic GABA neurotransmission which is important for normal information processing in the cerebellar system (Vicini & Ortinski, 2004). An ataxic response to the benzodiazepine diazepam is observed in α6 knockout mice, suggesting that the α6 subunit is important for directing myocoordination in benzodiazepine administration (Korpi et al., 1999). However, the α6 subunit does not alter sensitivity to ethanol and is not responsible for ethanol-induced motor impairment, demonstrated by no difference in rotarod performance after acute ethanol administration in C57Bl/6J α6 knockout and wild type mice (Korpi et al., 1999). The extrasynaptic alpha subunits 4-6 do not seem to contribute significantly to the symptoms associated with benzodiazepine and ethanol administration.

The presence of $GABA_A$ α-subunits 1-3 are correlated with the behavioral effects of benzodiazepines and ethanol. The α1 subunit is expressed in the thalamic and hippocampal region (Vicini & Ortinski, 2004). Many studies have been conducted with α1 knockout mice.

There is a notable lack in the developmentally regulated decrease in decay time for synaptic GABA currents in α1 knockout mice, suggesting that this subunit is necessary for the attenuation of GABA transmission in the cerebellar and hippocampal neurons (Vicini & Ortinski, 2004). After acute ethanol administration, α1 knockout mice exhibit increased locomotor activity (Blednov et al., 2003a). For high ethanol concentrations (>10% v/v), α1 knockouts have decreased consumption and preference, although reduced saccharin consumption suggests nonspecificity for this effect (Blednov et al., 2003a; Blednov et al., 2003b). A mutant model for benzodiazepine insensitivity was developed by Rudolph *et al.* (1999) where a knock-in point mutation at position 101 of the α1 subunit gene made α1 subunit-containing $GABA_A$ receptors insensitive to allosteric modulation by benzodiazepines. Behavioral studies with these mutants showed that the α1 subunit is correlated with the sedative, amnesic, and anticonvulsant properties of benzodiazepines (Vicini & Ortinski, 2004). The α2 and α3 subunits also have been demonstrated to effect the action of benzodiazepines and ethanol at the $GABA_A$ receptor. Through studies with various α subunit knock-in mice, the anxiolytic effects of benzodiazepines are correlated with functioning α2 subunits but not the α1, α3, and α5 subunits (Rudolph *et al.* 2012). The loss of righting reflex by combined application of ethanol and benzodiazepines is dependent on the α1 and α2 subunits (Blednov et al., 2003a; Vicini & Ortinski, 2004). Studies with α2(H101R) knock-in mice show that the α2 subunit is also associated with myorelaxative effect of diazepam (Crestani, Mohler, & Rudolph, 2001).

There are two major types of synaptic GABAA receptors in the CNS: the α2/ α3 subunits are often combined with the β3 subunit, and the α1subunit is often combined with the β2 subunit (Vicini & Ortinski, 2004). The α2/ α3/β3 receptor displays slow kinetics, which suggests that a slow-decaying inhibitory postsynaptic current allows for stronger and longer lasting inhibition

(Vicini & Ortinski, 2004). Since benzodiazepines affecting α2/ α3 subunits show anxiolytic effects, the longer lasting inhibition associated with the slow kinetic α2/ α3/β3 receptor could be correlated with decreased vigiliance and generalized anxiety (Vicini & Ortinski, 2004).

Benzodiazepines have been shown to have a receptor site on the $GABA_A$ receptor and ethanol has demonstrated positive modulatory effects that interact with different $GABA_A$ subunits. Steroid hormones produced in the brain were discovered to induce rapid effects by acting at receptor sites, as opposed to slow steroid action via crossing the blood brain barrier and acting at the genomic level (Belelli & Lambert, 2005). The mechanism of action for the rapid effects of neurosteorids remained unknown until the 1980s, when the neurosteroid 3α-hydroxy-5α-pregnan-20-one (allopregnanolone) demonstrated positive allosteric modification of the $GABA_A$ receptor (Belelli & Lambert, 2005).

Allopregnanolone

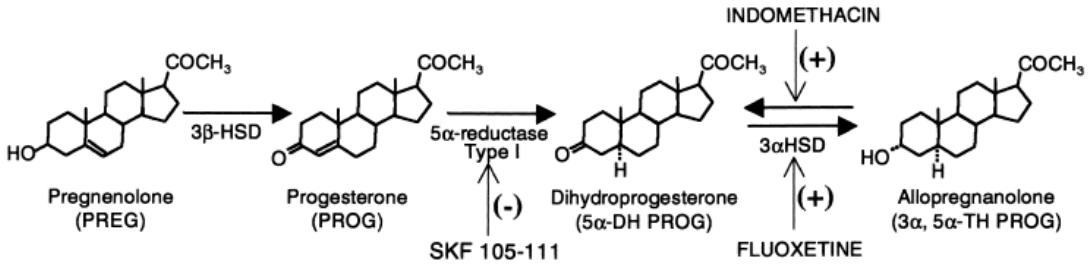
Function

Allopregnanolone, synthesized from the steroid progesterone, is a positive allosteric modulator of the $GABA_A$ receptor (Dong et al., 2001). Testosterone, estradiol, or glucocorticoids are not the major products of progesterone conversion in the brain, unlike the peripheral endocrine activity of progesterone (Guidotti et al., 2001b). Allopregnanolone is increased in the brain in stress paradigms, and also fluctuates naturally during the estrus cycle and pregnancy (VanDoren et al., 2000).

Synthesis

The main pathway for progesterone in the brain is the reduction to 5α-dihydroprogesterone (5α-DHP) by the enzyme 5-alpha-reductase (Dong et al., 2001). 5α-DHP is then reduced to allopregnanolone by the enzyme 3α-hydroxysteroidoxidoreductase (3α-HSOR)

(Dong et al., 2001). Allopregnanolone can also be oxidized by 3α-HSOR and converted back to 5α-DHP, seen in the figure below (Dong et al., 2001).

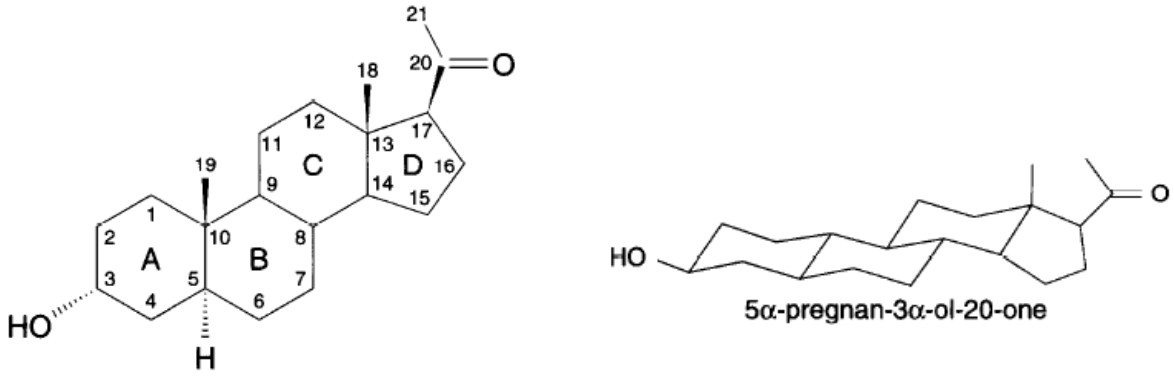


SKF 105-111: 17β-17[bis (1-methyl) amino carbonyl]androstane-3,5-dilene-3-carboxylic acid
3βHSD: 3β-hydroxysteroid dehydrogenase isomerase
3αHSD: 3α–hydroxysteroidoxidoreductase

Allopregnanolone synthetic pathway (Guidotti et al, 2001)

Action at the $GABA_A$ Receptor

Inhibitory neurotransmission by $GABA_A$ receptors undergoes positive modulation by endogenous allopregnanolone activation (Puia, Ducic, Vicini, & Costa, 1993; Hosie, Wilkins, da Silva, & Smart, 2006). GABAergic transmission is modulated by allopregnanolone via nongenomic action where the neurosteriod binds to a postulated specific allosteric center on the $GABA_A$ receptor that is separate from the binding site of benzodiazepines and barbiturates (Dong et al., 2001; Lambert, Belilli, Hill-Venning, & Peters, 1995). In contrast, the neurosteroid 5α-DHP regulates GABA transmission via genomic regulation of DNA transcription for $GABA_A$ subunits after binding to intracellular progesterone receptors (Dong et al., 2001). The chemical structure of allopregnanolone has a 5α- reduced androstane skeleton with an α-hydroxyl at the C3 position of the steroid A ring (Lambert et al., 1995). A keto group is also present at the C20 of the pregnane steroid side chain (Lambert et al., 1995). Agonist activity at the $GABA_A$ receptor requires the reduction of the 20-keto group and β configuration of the C17 side chain. (Lambert et al., 1995). The figures below illustrate the chemical composition of the neurosteroid:



Chemical structure of allopregnanolone (Lambert *et al.*, 1995).

Allopregnanolone increases the average open channel duration of the $GABA_A$ receptor by inducing a change in the proportion of GABA-gated channels that enter three kinetically distinct open states (Lambert et al., 1995). Direct binding of the neurosteroid to the receptor does not prolong single channel openings, but increases the probability that the channel will enter an open state of a relatively long duration (Lambert et al., 1995). Binding also increases the frequency of single channel openings, similar to the mechanism of action of benzodiazepines on the $GABA_A$ receptor (Lambert et al., 1995).

Behavior

The positive modulation of the $GABA_A$ receptor by allopregnanolone is crucial for normal GABAergic functioning. Animal models demonstrate how allopregnanolone deficiencies lead to increased anxiety and dysphoria, symptoms associated with human mood disorders. Socially isolated mice show a decrease in brain allopregnanolone levels, presumably due to the long term adaptation to the stress of social isolation (Dong et al., 2001). The mechanism for this down-regulation is linked to the 5α-reductase enzyme: mRNA and protein expression of 5α-reductase but not 3α-HSOR in the mouse brain is observable during the long-term social isolation (Guidotti et al., 2001a). Mice isolated for 4-6 weeks exhibit increased anxiety and

aggressiveness towards an intruder, elicited from a down-regulation in GABAergic efficiency due to the decrease of brain allopregnanolone expression (Dong et al., 2001; Guidotti et al., 2001). These mice also have resistance to the sedative action of indirect or direct GABAmimetic drugs, like pentobarbital (Guidotti et al., 2001). The long-term stress model is relevant to psychiatric symptoms associated with human mood disorders since a study with depressed patients also found an allopregnanolone deficiency (Guidotti et al., 2001). The beneficial use of fluoxetine to treat the depressive symptoms in these patients is postulated to be mediated by increased biosynthesis of allopregnanolone in lacking brain areas, which acts to facilitate GABA gating at the $GABA_A$ receptor (Guidotti et al., 2001).

Allopregnanolone also has anticonvulsant properties, demonstrated by protection against the convulsions induced by $GABA_A$ receptor antagonists in animals (Lambert et al., 1995). Allopregnanolone also acts as an allosteric inhibitor of the [35S]TBPS binding site on the picrotoxin receptor, suggesting that the neurosteroid has potent anticonvulsant properties (Belelli & Gee, 1989). Seizure disorders in humans are also associated with changing levels of progesterone; an increased frequency for seizures was seen in a subset women with catamenial epilepsy during menstruation when progesterone levels were low (Lambert et al., 1995). Allopregnanolone has demonstrated to have anxiolytic, sedative, and anticonvulsant properties that could have therapeutic applications in patients with mood disorders.

Allopregnanolone, Ethanol, and the $GABA_A$ Receptor

The pharmacological profile of allopregnanolone is similar to that of ethanol in regard to modulating the $GABA_A$ receptor (VanDoren et al., 2000; Finn et al., 2004). Allopregnanolone has demonstrated a mediating role for the effects of alcohol at the $GABA_A$ receptor (VanDoren et al., 2000). Studies demonstrate that after acute i.p ethanol administration in rats, levels of

allopregnanolone were dose- and time- dependently increased in the cerebral cortex (VanDoren et al., 2000). Levels of allopregnanolone also contribute to the hypnotic effects of ethanol, observed by a correlation between the ethanol-induced loss of righting reflex in rats and brain allopregnanolone levels (Van Doren et al., 2000). The anxiolytic effect of acute ethanol administration is also potentiated by exogenous administration of allopregnanolone, measured by increased open-arm time for mice in the elevated plus maze (Hirani, Sharma, Jain, Ugale, & Chopde, 2005). This confirms the anxiolytic effect of allopregnanolone at the $GABA_A$ receptor and demonstrates an indirect action of ethanol at the $GABA_A$ receptor by way of neurosteroid intermediaries (Hirani et al., 2005).

The 5α-reductase inhibitor finasteride blocks the anticonvulsant effect of ethanol on bicuculline induced seizure thresholds, suggesting that allopregnanolone has a role in mediating ethanol-induced convulsions (VanDoren et al., 2000). Ethanol-dependent rats are also sensitized to the anticonvulsant effect of allopregnanolone during ethanol withdrawal (Devaud, Purdy, Finn, & Morrow, 1996). Ethanol-induced increases in endogenous allopregnanolone levels may potentiate the effect of ethanol at the $GABA_A$ receptor (Finn et al., 2004). In C57Bl/6 mice with voluntary access to ethanol via a two bottle choice, consumption of ethanol significantly increased brain allopregnanolone levels in male but not female mice, even though females were shown to drink more ethanol on average (Finn et al., 2004). Interestingly, i.p injection of ethanol was not demonstrated to increase levels of allopregnanolone, suggesting that sex and mode of administration has an effect on endogenous allopregnanolone levels (Finn et al., 2004). The interactions of ethanol and allopregnanolone at the $GABA_A$ receptor suggests that endogenous levels of allopregnanolone may have a role in mediating susceptibility to alcohol abuse and withdrawal severity.

Alcohol and Anxiety

The anxiolytic effects of ethanol have been proposed to contribute to the initiation of alcohol drinking. Short-term acute ethanol consumption shows anxiolytic effects due to increases in GABAergic activity, while long term alcohol consumption produces anxiogenic effects in withdrawal possibly due to decreased GABAergic tone (Kushner, Abrams, & Borchardt, 2000). Humans with anxiety disorders are prone to self-medicate with alcohol (Stewart et al., 1995). Men and women who report to consume alcohol daily have shown to higher blood pressure, leading to the tension-reduction hypothesis for human consumption of alcohol (Klatsky & Gunderson, 2008). Moderation or reduction in amount of alcohol consumed is shown to reduce blood pressure in individuals with chronic daily alcohol intake (Klatsky & Gunderson, 2008). A study demonstrated that women with high anxiety sensitivity (defined as the extent to which an individual becomes fearful as a result of suspected consequences of anxiety symptoms) consume more alcohol and drink more often to excess than women with low anxiety sensitivity (Stewart et al., 1995).

In rodents, rats classified as anxiety prone after an initial anxiety reading in the elevated plus maze show higher intake and ethanol preference than rats characterized as less anxious (Spanagel et al., 1995). C57Bl/6 mice with a knockout δ-opiod receptor have an innate high anxiety phenotype (Roberts et al., 2001). This strain shows higher ethanol preference and higher rates of ethanol self-administration than wild type mice and demonstrates attenuated anxiety behavior after alcohol consumption in the light-dark transfer test (Roberts et al., 2001). A review by C.L Kliethermes (2005) demonstrated that in general, ethanol withdrawal increases anxiety-like behavior in rodent models for “anxiety” such as the elevated plus maze, the light/dark box,

and the open field. Both human and rodent behavioral profiles show a correlation between high anxiety and alcohol self-administration.

$GABA_A$ and Alcohol Consumption

Symptoms of alcohol withdrawal are also linked to the GABAergic pathway and are modified by neurosteroids. Mice that are physiologically dependent on ethanol are seizure prone in ethanol withdrawal. Studies show that activation of GABAergic pathways reduces the severity of these seizures, suggesting that GABAergic inhibition antagonizes the intensity of ethanol withdrawal symptoms (Goldstein, 1973). In studies using an ethanol vapor chamber to induce withdrawal symptoms in male mice, increased anxiolytic behavior was observed in the elevated zero maze 24 and 48 hours after removal from the vapor chamber (Kliethermes, Cronise, & Crabbe, 2004). Increased anxiety during alcohol withdrawal is also observed in humans, where clinical studies show alcoholics in extended withdrawal from alcohol have persistent hyperventilation and anxiety symptoms that do not decrease with the duration of abstinence (Roelofs, 1985). The negative symptoms associated with ethanol withdrawal are contributing causes leading to relapse of alcohol use.

Role of Sex Differences in Ethanol Consumption and Withdrawal

There are notable sex differences involving ethanol consumption and withdrawal severity. Female mice in ethanol withdrawal showed more anxiety-like behavior in the light-dark transition test than male mice, suggesting that females might have a more severe ethanol withdrawal profile than males (Kliethermes et al., 2004). In a study with intermittent ethanol self-administration, female C57Bl/6 mice consume more ethanol on average than male mice (Hwa et al., 2011). While females consume more than males, their level of ethanol preference does not differ significantly (Finn et al., 2004; Hwa et al., 2011). There are significant sex

differences in levels of allopregnanolone in the mouse brain after ethanol consumption. Voluntary consumption of ethanol in C57Bl/6 mice showed a decrease in brain allopregnanolone levels in male mice but not females (Finn et al., 2004). Interestingly, i.p injection of ethanol did not lead to any difference in endogenous allopregnanolone for male or female mice (Finn et al., 2004). The differences in brain allopregnanolone after ethanol consumption may have implications for susceptibility to alcohol abuse. Human sex differences for alcohol consumption contrast the sex differences seen in the C57Bl/6 strain: women and men differ little in the probability of drinking versus abstaining and men consistently consume more alcohol more frequently than women (Wilsnack et al., 2000). The discrepancies for alcohol consumption between sexes and across species are likely due to differences in the direct and indirect pharmacological mechanisms by which ethanol interacts in the brain.

Animal Mutant Model: $GABA_A$ subunit α2 knock-in induced allopregnanolone insensitivity

In order to further study the interactions of ethanol with the endogenous neurosteroid allopregnanolone at the $GABA_A$ receptor, an animal model for allopregnanolone insensitivity was developed by the Stephen Moss Lab at the Sackler School of Graduate Biomedical Sciences, Tufts University. *In vitro* studies demonstrated that the positive modulation at the $GABA_A$ receptor by allopregnanolone was abolished by replacing amino acids Gln 241 and Thr 236 along the α1 subunit transmembrane domain (TM1) with hydrophobic residues isoleucine and leucine (Hosie et al., 2006). These hydrophobic residues disrupt the hydrogen bonding of the hydroxyl and ketone groups of the neurosteroid with polar or charged bodies (Hosie et al., 2006). Potentiation by the neurosteroid was also abolished when Gln 241 in the $GABA_A$ α2 subunit was replaced with either leucine or methionine (Hosie, Clarke, da, & Smart, 2009). These studies show that allopregnanolone action at the $GABA_A$ receptor is greatly reduced when amino acids

in either the α1 or α2 subunits are substituted with hydrophobic residues. In theory, the hydrophobic substituted amino acids disrupt the action of the hydrophillic component of the polar ketone group of the allopregnanolone molecule. Furthermore, the knock-in mutation, rather than knockout, does not wholly eliminate the function of a specific gene but rather disrupts some DNA sequences. A knock-in mutation also limits the extent to which other subunit expression is unregulated due to a deletion of one specific subunit.

Objective

The aim of this study is to further analyze the effect of endogenous neurosteroid action at the $GABA_A$ receptor and how this action impacts characteristics of ethanol consumption and anxiety-like behavior. Using a C57Bl/6 mutant model for allopregnanolone insensitivity, male and female wild type, heterozygous, and homozygous animals for the mutation were studied for differences in ethanol consumption, withdrawal severity, and anxiety with and without ethanol withdrawal. If the knock-in $GABA_A$ α2 subunit mutation successfully inhibits allopregnanolone modulation of the $GABA_A$ receptor, then animals homozygous for the mutation should not show decreased levels of anxiety in behavioral tests when compared to control animals. For homozygous animals in withdrawal from ethanol, the interaction of allopregnanolone at the $GABA_A$ receptor should also not have an effect on decreased anxiogenic behavior. Heterozygous animals should show anxiolytic behavior that is intermediate between the homozygous and wild type activity. Neural concentrations of allopregnanolone have demonstrated to effect the expression of $GABA_A$ receptors and have shown to positively modulate the receptor via direct binding. Allopregnanolone has also been correlated with anxiolytic activity in ethanol withdrawal, suggesting a possible link between the regulatory action of the endogenous neurosteroid and ethanol via GABAergic mechanisms. Using the $GABA_A$ α2 allopregnanolone

insensitive mutants, the different genotypes for the mutation should reveal different behavioral effects for severity of alcohol withdrawal and level of anxiety-like behavior in open field and social preference tests. This study attempts to further understand the correlation between allopregnanolone and anxiolytic behavior, ethanol consumption, and withdrawal severity.

Methods and Materials

Subjects

Breeding pairs of C57B1/6J mice (Jackson Laboratory, Bar Harbor, Maine) were originally obtained from the Stephen Moss lab at the Sackler School of Graduate Biomedical Sciences and bred in Bacon Hall at Tufts University. All mice had a $GABA_A$ alpha-2 subunit knock-out mutation that induced insensitivity to the progesterone metabolite allopregnanolone. The reduced neurosteroid potentiation at the $GABA_A$ receptor was achieved by replacing Glutamine 241, located at the base of a cavity between the alpha 2 subunit's M1-M4 domains, with the hydrophobic (either leucine or methionine) (Hosie et al., 2006; Hosie et al., 2009) through adenoviral vectors (Lindemann & Schnittler, 2009).

The housing conditions for mouse pups and parents were maintained at 21.4° +/- 2.8° C and 20 +/- 5 % humidity. Mice were kept on a reverse light cycle where lights were turned off at 6am and turned on at 6pm in order to preserve nocturnal behavior for behavioral testing during the day. The subjects were weaned at 21 days postnatally and housed with their littermates, separated by gender, until postpartum day 60. Mice were then singly housed for the duration of the experiment in clear polycarbonate cages (28x17x14 cm) with pine shavings. Single-housed mice had free access to food (Purina, St. Louis, MO) and tap water provided above them in a stainless steel wire lid.

Female stimulus mice used for Social Preference testing were outbred adult Swiss-derived Carworth Farms Webster mice (CFW; Charles River Laboratories, Wilmington, MA). Female stimulus mice were initially group housed in large clear polycarbonate cages (17 X 48 X 13 cm) with corn cob bedding. Following ovariectomy surgery, each female was singly housed in the 28x17x14 cm clear polycarbonate cages with free access to food and water.

Animals were kept under conditions outlined by the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011).

Materials

Drugs

Solutions of alcohol were prepared each day of alcohol access from a stock solution of 95 w/v ethanol (Pharmco-Aaper, Brookfield CT, USA) and tap water. Ethanol was prepared in 3%, 6%, 10%, and 20% concentrations.

Open field and social preference tests

Allopregnanolone, a neurosteroid metabolite of progesterone (Steraloids, Inc., Newport, Rhode Island) was injected intraperitoneally in a 10 mg/kg dose (Fish, DeBold, & Miczek, 2002) and a 5.0 mg/kg dose. The vehicle for the neurosteroid was (2-hydroxypropyl)- β - cyclodextrin (Sigma-Aldrich Pharmaceuticals, LLC), prepared by dissolving the β-cyclodextrin in saline.

Midazolam challenge in the open field

The fast-acting benzodiazepine midazolam (Sigma-Adrich, Inc., St. Louis, MO) was injected intraperitoneally in a 0.56 mg/kg dose (Nunes-de-Souza et al., 2000; Lalonde & Strazielle, 2010). The vehicle for midazolam was saline, 0.9% sodium chloride (Baxter Healthcare Corporation, Deerfield IL, USA).

Recording Devices

Bottles used for the Alcohol Intermittent Access protocol were weighed on a compact digital scale in order to measure daily amount of fluid intake. Ethovision 2.4 and XT video tracking software by Noldus (Noldus Information Technology, Wageningen, Netherlands) was used to track locomotion for the Open Field test. A solid state camera (Cohu, Inc., Poway, CA) was mounted overhead to record subject behavior for both anxiety tests. The camera was connected to a Dell computer running Windows 7 (Microsoft). The tracking software used a subtraction method of detection from a reference image taken of the open field arena in order to track the movement of the subject. The software measured locomotion through total distance travelled and thigmotaxis through duration spent in a center zone of the arena defined by the researcher.

Sociability and anxiolytic behavior in the Social Preference test was recorded using Ethovision Observer XT software by Noldus (Noldus Information Technology, Wageningen, Netherlands). The same solid state camera (Cohu, Inc., Poway, CA) was mounted overhead in order to visualize and record activity. The camera was connected to a Dell computer running Windows 7 (Microsoft). Total locomotion, time spent in each chamber, and total interaction time with the novel female or the empty cage was recorded. The tracking software measured nose-contact interactions as well as tracked the body of the subject through determining its center of gravity. The software tracked the subject by using a reference analog image from the camera taken prior to entering subjects into the test arena. The motion of the subject was tracked using a static subtraction method where the live analog image was subtracted from the reference image in order to determine location. Samples were recorded at a rate of 1 sample per second.

Intermittent Alcohol Access Protocol

Animals were given free access to two 50 mL water bottles. Food was evenly distributed on either side of the water bottles, and the placement of the alcohol-containing bottle was switched from the left to the right side with every day of alcohol access in order to control for side preference (Hwa et al., 2011). Alcohol was provided every other day, with the first day of access containing a 3% ethanol solution. Concentration was increased to 20% ethanol after three alcohol access days and animals were maintained on the intermittent drinking protocol for at least 8 weeks. A "drip cage" served as a control for the amount of water or alcohol that was lost due to leaking sipper tubes or evaporation. The drip cage was an empty clear polycarbonate cage (28x17x14 cm) with pine shavings and the same stainless steel wire lid. The control drip bottles were handled and weighed in the same manner as the experimental bottles. Bottles were weighed in the morning from 8:30am to 9:30am, three hours after the end of the light cycle. Body weight for each subject was measured on the day of alcohol access in order to calculate the amount of alcohol consumption in grams per kilogram of body weight.

Handling Induced Convulsion Scores

After 8 weeks of intermittent alcohol access, subjects were tested for severity of alcohol withdrawal by performing a handling induced convulsion (Goldstein, 1972). Subjects were tested every two hours since the removal of access to alcohol for 10 hours. The animal was lifted by the tail and suspended in the air for a few seconds and observed for characteristics of a tonic or a tonic-clonic convulsion. The activity of the suspended mouse was scored on a 0-4 scale were 0 was no convulsion and 4 was a violent tonic-clonic convulsion that continued after the mouse was released back to its home cage.

Ovariectomy Surgery

CFW females underwent ovariectomy surgery in order to act as stimulus animals for the C57Bl/6 subjects in the Social Preference paradigm. Each female subject was injected i.p. with a combination of ketamine (100 mg/kg) and xylazine (10 mg/kg) and a 50 mg/kg dose of carpofen subcutaneously 5 minutes prior to surgery. A small incision was made on the left lateral and right lateral sides of the subject, and the ovary was lifted out by the fallopian tube with sterile blunt tip tweezers. The end of the ovary was tied off with sterile surgical thread and then removed with scissors. A wound clip was applied to the outermost layer of skin and the subject was returned to its home cage. A subcutaneous injection of 50 mg/kg carpofen was injected once per day for three days post surgery. After one week the wound clips were removed from both sides of the subject.

Tests for Anxiolytic Behavior

Open Field Test

The Open Field Test (C. S Hall, 1936; Wilcock & Broadhurst, 1967; Denenberg, Gartner, and Myers, 1975) was used to measure the level of thigmotaxis in the allopregnanolone insensitive C57Bl/6 mice. Four plastic tubs (47x28x47 cm) were aligned on the ground in a 2x2 configuration under an overhead mounted camera. The camera recorded the locomotion of the subject initially using Ethovision Observer 2.4 software by Noldus. The tracks recorded by this software were disregarded and redone using Ethovision Observer XT in order to ensure correct calibration across all Open Field tracks. A designated center zone was specified in the Ethovision program. The total distance travelled, the frequency entering the center zone, and the total duration in the center zone was measured over the course of 60 minutes. After each experimental

session, the open field was washed out with soap and water and dried to remove any residual odors.

Social Preference Test

The Social Preference Test was used to test each subject for the level of sociability (Moy et al., 2004) and anxiety (Yang, Clarke, & Crawley, 2009). Each subject was introduced to a three-chambered plastic box (63x42x23 cm), sectioned off into three equal chambers (21x42x23 cm) by clear plastic walls. Each chamber was accessible through an open doorway (8x10 cm). The subject was introduced to the center chamber and allowed to explore the box's empty compartments for 10 minutes, after which the animal was confined to the center chamber by placing a clear, polycarbonate box (28x17x14 cm) on top of the subject to restrict access to the other chambers. Two small, circular wire cups (diameter = 10 cm) acted as cages and were positioned in the box, one in each of the left and right chambers. An ovariectomized CFW female was placed under one of the cages and the other was left empty. The subject was then released from under the polycarbonate box and allowed free access to either the chamber with the empty cage, or the chamber containing the OVX female. The subject was allowed 10 minutes to explore the three chambers freely and movement was recorded by Ethovision Observer XT video tracking software by Noldus. Amount of interaction with the novel OVX female, the duration and frequency entering each chamber, and the total amount of locomotion was recorded. The females were sensitized to the wire circular cage for 30 minutes prior to test days. After each test session, the three-chambered box was cleaned with soap and water and dried to remove any residual odors.

Midazolam Challenge

Subjects were tested in the Open Field to measure locomotion and anxiolysis in response to an i.p injection of midazolam. Subjects were either given an injection of 0.56 mg/kg midazolam or the vehicle, saline. No subject was tested more than twice in the open field and a minimum of three days was required between test days in order to minimize familiarity with the open field environment. Ethovision Observer XT software tracked the center-point of each subject in the open field. The open field was washed out with soap and water prior to testing each subject.

Experiment 1: Intermittent Alcohol Access in the F2 Generation (Heterozygote and Wild Type)

Male (n = 16) and female (n = 8) C57Bl/6 $GABA_A$ alpha 2 knock in allopregnanolone insensitive mutants were administered alcohol according to the Intermittent Alcohol Access protocol for eight weeks. The mutants were heterozygous (male n = 9, female n = 5) or wild type (male n = 7, female n = 3) for the allopregnanolone insensitivity mutation. On the last alcohol access day of Week 8, all subjects were tested for severity of alcohol withdrawal using the Handling Induced Convulsion (HIC) score. The subjects were kept on the Intermittent Alcohol Access protocol and tested in the Open Field, Social Preference, and Midazolam Challenge in the Open Field during periods of peak alcohol withdrawal (approximately 4 to 8 hours into withdrawal). The subjects were verified for stability of alcohol drinking with a two-way repeated measure ANOVA after the beginning of Open Field and Social Preference testing.

Experiment 2: Intermittent Alcohol Access in the F3 Generation (Homozygote, Heterozygote, and Wild Type)

Male (n = 7) and female (n = 7) C57Bl/6 $GABA_A$ alpha 2 knock in allopregnanolone insensitive mutants were administered alcohol according to the Intermittent Alcohol Access protocol for eight weeks. The mutants were homozygous (male n = 2, female n = 1), heterozygous (male n = 3, female n = 5) or wild type (male n = 2, female n = 1) for the allopregnanolone insensitivity mutation. After eight weeks of intermittent alcohol drinking, the HIC scores for each animal every two hours into alcohol withdrawal was determined. Animals continued on the intermittent alcohol access protocol and were tested in the Open Field, Social Preference, and Midazolam Challenge in the Open Field during periods of peak alcohol withdrawal.

Experiment 3: Two-Bottle Choice Controls (Heterozygote and Wild Type)

Male (n = 12) and female (n = 4) C57Bl/6 $GABA_A$ alpha 2 knock in allopregnanolone insensitive mutants were administered water in each bottle of the Intermittant Alcohol Access protocol for eight weeks. These animals had no prior history with alcohol consumption and were never introduced to alcohol. The mutants were heterozygous (male n = 4, female n = 2) and wild type (male n = 8, female n = 2) for the allopregnanolone insensitive mutation. After eight weeks of water-drinking in the Intermittent Alcohol Access protocol, HIC scores for each subject were determined for the same time points as the animals in alcohol withdrawal. Subjects were tested in the Open Field, Social Preference, and Midazolam Challenge in the Open Field for the same time periods as the alcohol-drinkers. Animals continued to have water in the two-bottle choice for the duration of testing.

Statistics

Descriptive statistics and two-way repeated measure ANOVA tests completed using SigmaStat 3.1 (Systat Software, Inc. Point Richmond, CA). Non-parametric handling-induced convulsion scores were analyzed using a Mann-Whitney test using SigmaStat 3.1 software. ANOVA tests were followed by Bonferroni post hoc t-tests when appropriate. α was set at 0.05

Results

Table 1 shows the number of subjects for each treatment group.

Alcohol Consumption and Withdrawal Severity

Male (n = 23) and female (n = 15) C57Bl/6 $GABA_A$ α2 allopregnanolone insensitive mice both displayed a significant dose-dependent effect for ethanol concentration across all genotypes, consuming more ethanol with increasing ethanol concentration ($F(3, 36) = 129.49$, $p < 0.001$). Females consumed significantly more 20% ethanol than males across all genotypes for all eight weeks of 20% ethanol access ($F(1, 36) = 70.84$, $p < 0.001$) (See Figures 1 and 2). Average ethanol consumption for female homozygote mice across all genotypes was 30.79 g/kg (*SD* = 0.59) and 19.42 g/kg for males (*SD* = 0.31). Average ethanol preference 64.6 % (*SD* = 1.06) for females and 59.4% (*SD* = 0.10) for males. Male and female subjects in withdrawal from ethanol had a trend for higher median HIC scores than water-drinking controls in hour 6 of ethanol withdrawal ($t = 308.00$, $p = 0.013$) (See Figures 3 and 4). There was no statistically significant difference in ethanol consumption after the start of open field and social preferences tests ($F(4, 35) = 1.290$, $p = 0.277$).

The Open Field: Total Locomotion and Time in the Center Zone

The first five minutes of time spend in the open field was analyzed as opposed to the full 60 minute trial. Wild type control males with an i.p saline injection showed a consistent amount of time in the center zone of the open field for each 10-min time bin; no time bin was significantly different than the others.

There was a main effect of ethanol experience on total locomotion in the open field across sex and genotypes ($F(1, 52) = 5.99$, $p = 0.018$) when allowing for difference in drug treatment. Animals in withdrawal from alcohol had significantly less mean total locomotion (cm) ($M = 1495.54$, $SD = 77.13$) than control animals ($M = 1779.23$, $SD = 85.24$) (See Figure 5). Sex also had a significant main effect on total locomotion across ethanol treatment and gentoypes ($F(1, 52) = 4.367$, $p = 0.041$) when allowing for differences in drug treatment (See Figures 6 and 7). Males displayed greater average locomotion (cm) ($M = 1681.95$, $SD = 78.51$) than females (cm) ($M = 1423.15$, $SD = 102.60$) when allowing for differences in drug treatment.

There was a significant main effect for sex across genotype and ethanol treatment animals in time spent in the center zone of the open field ($F(1, 52) = 5.15$, $p = 0.027$) when allowing for differences in drug treatment. Males spent more time in the center zone (s) ($M = 178.41$, $SD = 7.79$) than females (s) ($M = 150.65$, $SD = 10.18$) (See Figures 8 and 9).

There was a main significant interaction for drug treatment and ethanol experience across genotypes and sex ($F(4, 52) = 4.647$, $p = 0.001$) for time spend in the center zone. Post-hoc tests showed that the 10.0 mg/kg allopregnanolone dose was significantly different for control animals versus those in withdrawal from ethanol ($t = 2.77$, $p = 0.006$). Control animals spent significantly more time in the center zone (s) ($M = 209.57$, $SD = 11.58$) than animals in ethanol withdrawal (s) ($M = 154.043$, $SD = 11.26$) for the 10.0 mg/kg allopregnanolone dose. For the

control animals across sex and genotype, there were significant differences between the drug treatments ($F(4, 52) = 2.51$, $p = 0.044$). Post-hoc tests revealed that the 10.0 mg/kg dose was significant for time spend in the center zone compared to the saline, midazolam, and 5.0 mg/kg allopregnanolone doses ($t = 3.34$, $p = 0.011$; $t = 3.86$, $p = 0.002$; $t = 3.32$, $p = 0.011$, respectively). The 10.0 mg/kg increased time spend in the center zone (s) ($M = 209.57$, $SD = 11.58$) compared to saline (s) ($M = 154.87$, $SD = 11.58$), 0.56 mg/kg midazolam (s) ($M = 146.45$, $SD = 11.58$), and 5.0 mg/kg allopregnanolone (s) ($M = 155.27$, $SD = 11.58$).

Difference in genotype had no significant main effect on total locomotion or time spent in the center zone of the open field ($F(2, 51) = 2.56$, $p = 0.088$; $F(2, 51) = 0.0098$, $p = 0.990$, respectively). For control males, there was a significant effect of genotype on total locomotion ($F(1,10) = 9.97$, $p = 0.010$). Post-hoc tests revealed that there was a significant difference in locomotion between wild type and heterozygous males ($t = 3.16$, $p = 0.010$). Wild type ethanol naive males showed greater average locomotion (cm) ($M = 1966.13$, $SD = 96.92$) than heterozygous males ($M = 1436.00$, $p = 137.06$). Control males also showed a significant genotype difference for time spend in the center of the open field ($F(1,10) = 8.267$, $p = 0.017$). Post-hoc tests showed a significant difference between wild type and heterozygous males for time in the center zone ($t = 2.88$, $p = 0.017$). Wild type control males spend significantly more (s) ($M = 192.39$, $SD = 9.30$) time in the center zone than heterozygous males ($M = 146.07$, $SD = 13.16$).

Control males also showed significant differences in drug treatment for time spend in the center of the open field ($F(4,10) = 7.11$, $p < 0.001$). Post-hoc tests show that both wild type and heterozygous males had significant differences in time spend in the center zone for the 10.0 mg/kg dose of allopregnanlone versus the midazolam dose, the saline dose, and the 5.0 mg/kg

dose of allopregnanolone (t = 4.48, p <0.001; t = 4.24, p = 0.001; t = 3.91, p = 0.003, respectively). Time spent in the center zone for the 10.0 mg/kg dose of allopregnanolone was significantly higher (s) (M = 218.42, SD = 11.73) than the midazolam (s) (M = 144.00, SD = 11.73), saline (s) (M = 148.03, SD = 11.73), and 5.0 mg/kg dose of allopregnanolone (s) (M = 153.53, SD = 11.73) for both wild type and heterozygous control males. Post-hoc tests for wild type control males reveal significant differences between drug treatments. The 10.0 mg/kg allopregnanolone dose significantly increased time spent in the center zone (s) (M = 246.01, SD = 13.55) over the midazolam dose (s) (M = 147.19, SD = 13.56), the saline dose (s) (M = 176.94, SD = 13.56), and the 5.0 mg/kg dose (s) (M = 183.74, SD = 13.56) (t = 5.16, p <0.001; t = 3.60, p = 0.009; t = 3.25, p = 0.023, respectively).

Social Preference

The time the subject spent in the novel OVX female zone of the social preference arena was compared for different drug treatments, ethanol experience, sex, and genotype. Main effects for differences in drug treatment were statistically significant for time spent in the novel OVX female zone (F(2, 52) = 4.59, p = 0.014) (See Figures 10 and 11). Post-hoc tests revealed differences for the 10.0 mg/kg dose of allopregnanolone compared to the 5.0 mg/kg dose and vehicle (t = 2.76, p = 0.023; t = 2.63, p = 0.032, respectively). The 10.0 mg/kg dose increased the time spent interacting with the novel OVX female (M = 149.90, SD = 15.12) than the 5.0 mg/kg allopregnanolone dose (M = 91.47, SD = 14.81) and the vehicle injection (M = 93.61, SD = 15.13).

Ethanol experience had a significant main effect on time spent interacting with the novel OVX female (F(1, 52) = 10.02, p = 0.003). Post-hoc test showed a significant difference for the

mean OVX interaction time between control and withdrawal animals (t = 3.17, p = 0.003). The mean interaction time for control animals was greater (s) (M = 160.98, SD = 16.41) than animals in ethanol withdrawal (s) (M = 97.42, SD = 11.53). Drug treatment also had a significant main effect for difference in time spent in the novel OVX zone (F(2, 52) = 4.39, p = 0.017) while allowing for differences in ethanol experience. Post-hoc tests show a difference in drug treatment for the 10.0 mg/kg allopregnanolone dose versus the vehicle injection (t = 2.82, p = 0.019). The 10.0 mg/kg allopregnanolone dose significantly increased interaction time (s) (M = 165.211, SD = 14.66) compared to the vehicle injection (s) (M = 107.32, SD = 14.48).

There were also significant main effects for differences in interaction time for the 5.0 mg/kg allopregnanolone injection and the 10.0 mg/kg injection between control and withdrawal animals (t = 2.51, p = 0.013; t = 2.32, p = 0.022, respectively). The 5.0 mg/kg allopregnanolone injection significantly increased OVX interaction time for the control animals (s) (M = 159.03, SD = 27.27) versus the 5.0 mg/kg dose in the withdrawal animals (s) (M = 71.12, SD = 16.70). The 10.0 mg/kg dose also significantly increased OVX interaction time for control animals (s) (M = 202.33, SD = 23.10) than the 10.0 mg/kg injection for animals in withdrawal (M = 128.05, SD = 18.06).

There were no significant main effects for the different genotypes on time spent interacting with the novel OVX female (F(2, 51) = 0.157, p = 0.855). However, there was a trend for the heterozygous and homozygous male and female animals in withdrawal to increase interaction time for the vehicle injection when compared to wild type animals in withdrawal (Figure 12). For control males, there were significant effects of genotype (F(1, 10) = 5.18, p = 0.044) and drug treatment (F(2, 10) = 6.60, p = 0.010). Post-hoc tests reveal that the effect for genotype within control males was not significant (t = 2.18, p = 0.054). Post-hoc tests for drug

treatment reveal that the 5.0 mg/kg (t = 3.11, p = 0.023) and 10.0 mg/kg (t = 2.78, p = 0.044) dose are both significantly different than the vehicle injection for wild type and heterozygous males. The 5.0 mg/kg (s) (M = 216.52, SD = 22.76) and the 10.0 mg/kg (s) (M = 196.14, SD = 17.68) both increased interaction time over the vehicle (s) (M = 130.51, SD = 15.68) for wild type and heterozygous males.

Discussion

The C57Bl/6 $GABA_A$ α2 allopregnanolone insensitive mice displayed significant sex and ethanol treatment differences in the intermittent alcohol access protocol, open field, and social preference tests. Ethanol experience was found to have a significant effect for differences in total locomotion in the open field, time spent in the center zone, and time interacting with a novel mouse in the social preference test. Significant sex differences were also found for total locomotion and time in the center zone for the open field test. Differences in genotype for the $GABA_A$ α2 allopregnanolone insensitive mutants did not have an effect for ethanol consumption, time spend in the center of the open field, nor time spent interacting with a novel female in the social preference test.

Ethanol Consumption and Withdrawal Severity

The intermittent alcohol access protocol is a clinically relevant animal model for ethanol consumption as it is voluntary and successfully demonstrates heavy episodic “binge” drinking (Hwa et al., 2011). Female C57Bl/6 $GABA_A$ α2 allopregnanolone insensitive mice displayed greater ethanol consumption over nine weeks of intermittent ethanol access, despite there being no difference for alcohol preference between males and females. These results confirm the sex differences in ethanol consumption for the C57Bl/6 strain (Hwa et al., 2011). There were no

significant differences between each genotype for level of alcohol consumption for both males and females, suggesting that initiation of ethanol consumption is not mediated by action of allopregnanolone at the GABA-A receptor. Homozygous, heterozygous, and wild type animals consumed consistent amounts of 20% ethanol over the period of intermittent alcohol access and there was no effect on the start of open field and social preference testing on the amount of ethanol consumption.

Animals in withdrawal had higher median HIC scores, although wild type males and females as well as heterozygous males in the control group displayed low levels for seizing, suggesting that this mutant strain is slightly seizure prone. Homozygous female and male mice for the allopregnanolone insensitive mutation displayed greater median HIC scores than the heterozygous and wild type animals. This convulsion-prone phenotype in the homozygous mice provides evidence for the anticonvulsant properties of allopregnanolone for animals in ethanol withdrawal (Devaud et al., 1996; VanDoren et al., 2000). Animals without functional GABA inhibition via allopregnanolone action at the GABA-A receptor demonstrate a more severe withdrawal symptom profile.

Anxiety-like Behavior in the Open Field

Animals that were never exposed to ethanol were more active in the open field and spent more time in the center zone when compared to animals tested in withdrawal from ethanol. This discrepancy between the control and withdrawal animals confirms findings that alcohol withdrawal develops a more anxiogenic behavioral profile (Valdez et al., 2002). However, animals in withdrawal from ethanol also displayed less total activity in the open field as measured through locomotion. This decrease in general activity could confound the correlated

decrease in time spent in the center zone. For the 10.0 mg/kg dose of allopregnanolone, control animals displayed more anxiolytic effects in the open field, measured by their reduced level of thigmotaxis (Treit & Fundytus, 1989). This drug effect demonstrates that the anxiolytic properties of allopregnanolone are conserved in these mutants, including animals homozygous for the mutation.

Males also showed more exploratory behavior than females, demonstrated by their increased locomotion for all genotypes in the withdrawal and control groups as well as increased time in the center of the open field. Females did not show significantly greater activity than males for any drug dose in any genotype, despite other studies showing increased activity for female mice during estrus (Fernandez-Guasti & Picazo, 1992). The lack of anxiolytic behavior in the females could be attributed to a strain effect, as a study by Meziane *et al* (2006) showed that C57Bl/6 females do not display an estrous cycle effect for anxiety-like behaviors in the open field.

For male and female animals in the control group, there were significant drug treatment effects for the 10.0 mg/kg allopregnanolone dose when compared to saline, midazolam, and 5.0 mg/kg allopregnanolone doses. The 10.0 mg/kg dose significantly increased time spent in the center zone when compared to these other treatments. There was not a significant difference between the vehicle injection (20% B-cyclodextrin) and the 10.0 mg/kg allopregnanolone dose; however a trend is apparent for the 10.0 mg/kg dose increasing time spent in the center zone compared to the vehicle injection. Overall, the 10.0 mg/kg dose for the control animals has an anxiolytic effect as measured by increased time spent in the center zone of the open field. The lack of significant drug effects in the withdrawal group could be due to the interaction of ethanol at the $GABA_A$ receptor with the neurosteroid. The male homozygous animals in ethanol

withdrawal (n = 2) display a trend where increasing dose of allopregnanolone decreases time spend in the center zone of the open field, suggesting that these animals may have intact allopregnanolone insensitivity *in vivo*.

Male animals with no history of ethanol access show significant genotype effect on total locomotion in the open field and time spend in the center of the open field. Wild type males tended to display more activity in the arena than heterozygote males and wild type males displayed more time spend in the center zone. These data suggest that control wild type males show more anxiolytic behavior than heterozygous males, suggesting that intact allopregnanolone receptor sites on the GABAA receptor are necessary for reducing anxiety-like behaviors.

The midazolam challenge showed no significant effects for the 0.56 mg/kg dose for males and females in withdrawal and with no ethanol history. Studies have suggested decreased sensitivity to benzodiazepines and heighted anxiogenic responses after acute exposure to allopregnanolone is due to an increased expression of the $GABA_A$ $\alpha4$ receptor subunit (Gulinello & Smith, 2003). The $\alpha2$ subunit has also been shown to mediate benzodiazepine anxiolytic effects (Birzniece et al., 2006) and this knock-in mutation at the $\alpha2$ subunit may also have an effect on benzodiazepine binding efficacy.

Anxiolytic Effects in the Social Preference Test

Time that the subject spent interacting with a novel ovariectomized CFW female was dependent on ethanol history, but not sex. Significant drug treatment effects were found across both male and female groups, but there was no significant interaction between sex and drug treatment. The 10.0 mg/kg allopregnanolone dose increased the time that control and withdrawal male and female animals spent interacting with the novel mouse, illustrating an anxiolytic effect

for allopregnanolone regardless of an ethanol interaction. An interaction was also found for ethanol experience and drug treatment: the 5.0 mg/kg and 10.0 mg/kg dose of allopregnanolone significantly increased interaction time for control subjects compared to subjects in ethanol withdrawal.

The social preference test further illustrates the increased anxiogenic profile for animals in withdrawal from ethanol, previously discussed in the open field test. Homozygous male and female animals in withdrawal from ethanol had comparable interaction time to control male and female animals with no ethanol drinking history. This interaction demonstrates that the homozygous animals with complete allopregnanolone insensitivity have a less severe withdrawal profile than animals with intact allopregnanolone interaction at the $GABA_A$ receptor. This shows that allopregnanolone modulation of the GABAA receptor in withdrawal from ethanol increases the severity of the ethanol withdrawal as measured by a heightened anxiogenic profile. This test also demonstrated reduced "anxiety" at the 5.0 and 10.0 mg/kg allopregnanolone dose for control animals, showing allopregnanolone has anxiolytic effects of for animals without an ethanol-drinking history. The animals tested in the social preference test in withdrawal from ethanol did not show a significant dose-dependent anxiolytic effect for increasing allopregnanolone dose. The social preference test demonstrated different effects for allopregnanolone for animals with different drinking histories: animals in withdrawal from ethanol with complete allopregnanolone insensitivity had a less "anxious" profile than animals with intact allopregnanolone action. Animals with no drinking history showed that allopregnanolone had an anxiolytic effect with increasing dose of allopregnanolone administration.

Limitations and Further Directions

This study was limited by the number of mice that were homozygous for the C57Bl/6 $GABA_A$ α2 allopregnanolone insensitive mutation. One female homozygous mouse and two male homozygous mice were tested for alcohol consumption and anxiolytic activity in the open field and social preference test. The lack of homozygous mice in the control group and the low number of subjects for the withdrawal group indicate that further studies with these animals are necessary in order to further elicit the differences between the genotypes for the allopregnanolone insensitive mutation. The low number of subjects also has a confound for the repeated measures design of this experiment, in that repeated testing in the open field and social preference tests can lead to habituation to an environment that should be completely novel to the subject in order to obtain anxiogenic effects. C57Bl/6 mice have shown preference for novel stimulus mice only when in a completely novel environment (Pearson, Defensor, Blanchard, & Blanchard, 2010). Increased sample size, especially in the wild type and homozygous genotypes, would further demonstrate ethanol treatment, sex, and genotype differences for the allopregnanolone insensitivity mutation.

The positive effect for allopregnanolone in reducing ethanol withdrawal severity and anxiety-like behavior show that this study has clinical relevance. By further examining the effects of allopregnanolone for ethanol withdrawal symptoms, therapeutic neurosteroid-based ligands could be developed to alleviate withdrawal severity or anxiety disorders in humans. This may not have relevance for women with premenstrual dysphoric disorder, who interestingly have increased levels of allopregnanolone that are associated with heightened anxiogenic symptoms, contrary to the anxiogenic effect of allopregnanolone deficiencies in rodents and humans without this disorder (Gulinello & Smith, 2003).

The intermittent alcohol access protocol is a successful animal model for demonstrating "binge" alcohol consumption in humans. However the increased daily alcohol consumption in female C57Bl/6 mice is contrary to human behavior, where men have been shown to demonstrate a daily ethanol intake that was about double the daily intake of women, and men were more likely to be classified as heavy drinkers (Dawson & Archer, 1993; Vengeliene et al., 2008).

Future studies should include adding more doses to the midazolam challenge in order to determine whether the anxiogenic response at the 0.56 mg/kg dose is due to a sedative effect at that dose or benzodiazepine insensitivity due to upregulation of different $GABA_A$ receptor subtypes. Another useful drug challenge would be administration of finasteride to block 5α-reductase activity. A finasteride challenge could demonstrate a behavioral profile in allopregnanolone insensitive wild type mice that is analogous to the homozygous mice due to lack of allopregnanolone action at the $GABA_A$ receptor, either through lack of allopregnanolone binding at the receptor site or lack of circulating endogenous allopregnanolone. A neurosteroid antagonist, 3α, 5α-17-phenylandrost-16-en-3-ol, has been shown to have no effect on GABA-evoked responses or on enhancement by barbiturates and benzodiazepines, but has been shown to selectively antagonize the GABA-modulatory and GABA-mimetic effects of allopregnanolone neurosteroids (Belelli & Lambert, 2005). Studies using this steroid antagonist would be useful for determining the effect of allopregnanolone action on "anxiety" behavior in the wild type mutants compared to the homozygous mutants. Aggression studies would be another interesting behavioral condition in these mutant mice. A study by Fish *et al.*, (2001) demonstrated mice with an alcohol-heightened aggression profile had reduced aggression after administration of allopregnanolone. Aggression screening with these mutants could elicit basal aggression

differences between the genotypes, with possibly increased aggression in the mutants homozygous for allopregnanolone-insensitivity.

This study demonstrates that ethanol and allopregnanolone behavioral effects are mediated through a similar neural pathway that is likely to be related to action at the GABA-A receptor. A long-term (> 8 weeks) history of ethanol “binge” drinking possibly modulates the GABA-A receptor so that there is decreased sensitivity to anxiolytic substances such as allopregnanolone and benzodiazepines.

Table 1-5. Number of subjects for each treatment group.

1. Intermittent Alcohol Access

	Male- Alcohol Access	Female- Alcohol Access	Male –Water Drinking Control	Female- Water Drinking Control
Wild Type	9	4	8	2
Heterozygous	12	5	4	2
Homozygous	2	1	0	0

2. Open Field Test – Alcohol Drinkers

	Vehicle	0.5 mg/kg Allopregnanolone	10.0 mg/kg Allopregnanolone	Saline	0.56 mg/kg Midazolam
Male Wild Type	5	2	6	9	9
Male Heterozygous	6	3	6	10	10
Male Homozygous	2	2	1	2	2
Female Wild Type	1	1	3	4	4
Female Heterozygous	7	5	3	10	10
Female Homozygous	1	1	1	1	1

3. Open Field test – Water Drinkers

	Vehicle	0.5 mg/kg Allopregnanolone	10.0 mg/kg Allopregnanolone	Saline	0.56 mg/kg Midazolam
Male Wild Type	8	8	8	8	8
Male Heterozygous	4	4	4	4	4
Male Homozygous	0	0	0	0	0
Female Wild Type	2	2	2	2	2
Female Heterozygous	2	2	2	2	2
Female Homozygous	0	0	0	0	0

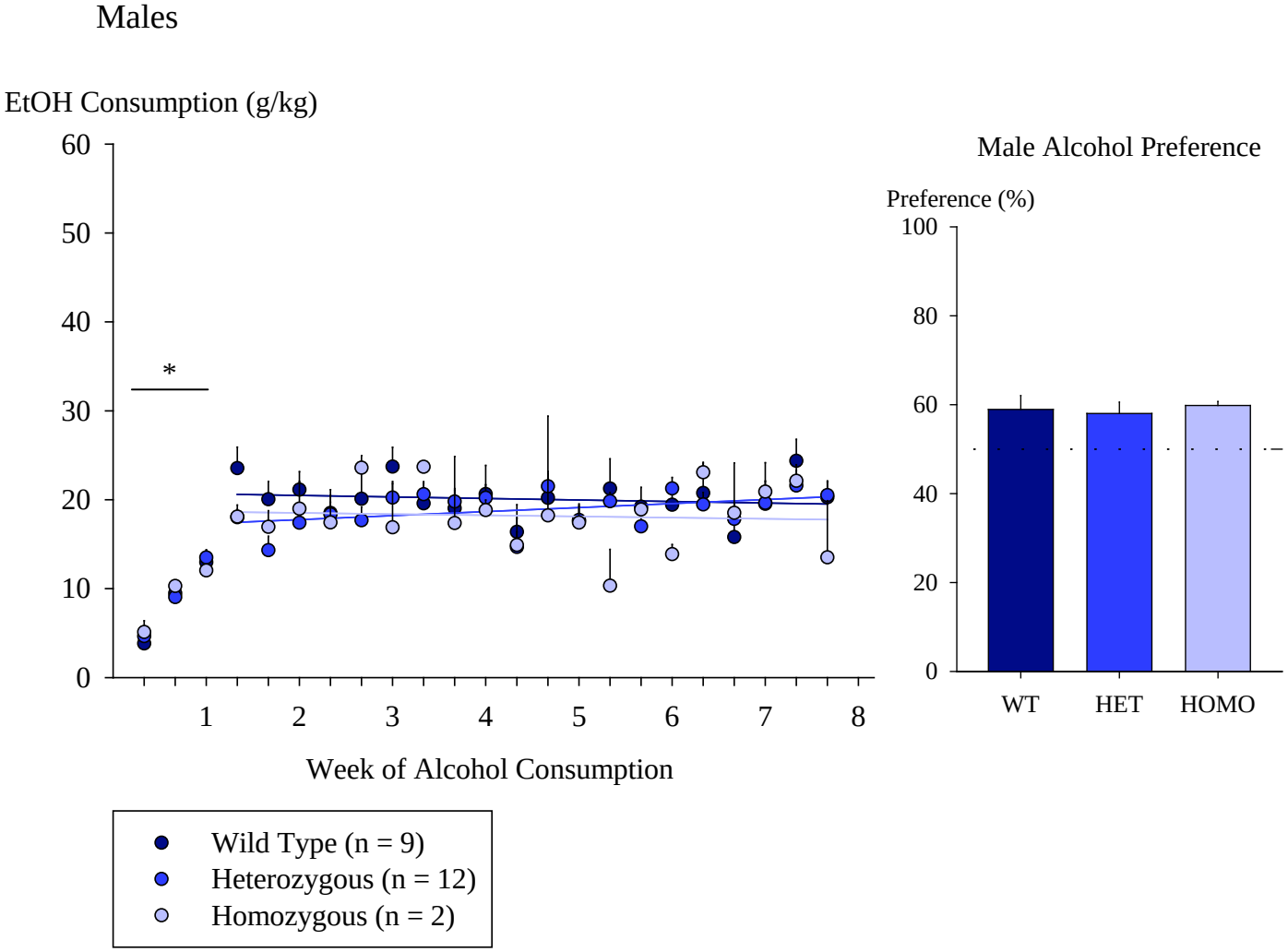
4. Social Preference Test- Alcohol Drinkers

	Vehicle	0.5 mg/kg Allopregnanolone	10.0 mg/kg Allopregnanolone
Male Wild Type	6	7	6
Male Heterozygous	8	8	8
Male Homozygous	1	2	2
Female Wild Type	1	3	3
Female Heterozygous	8	8	6
Female Homozygous	1	1	1

5. Social Preference Test – Water Drinkers

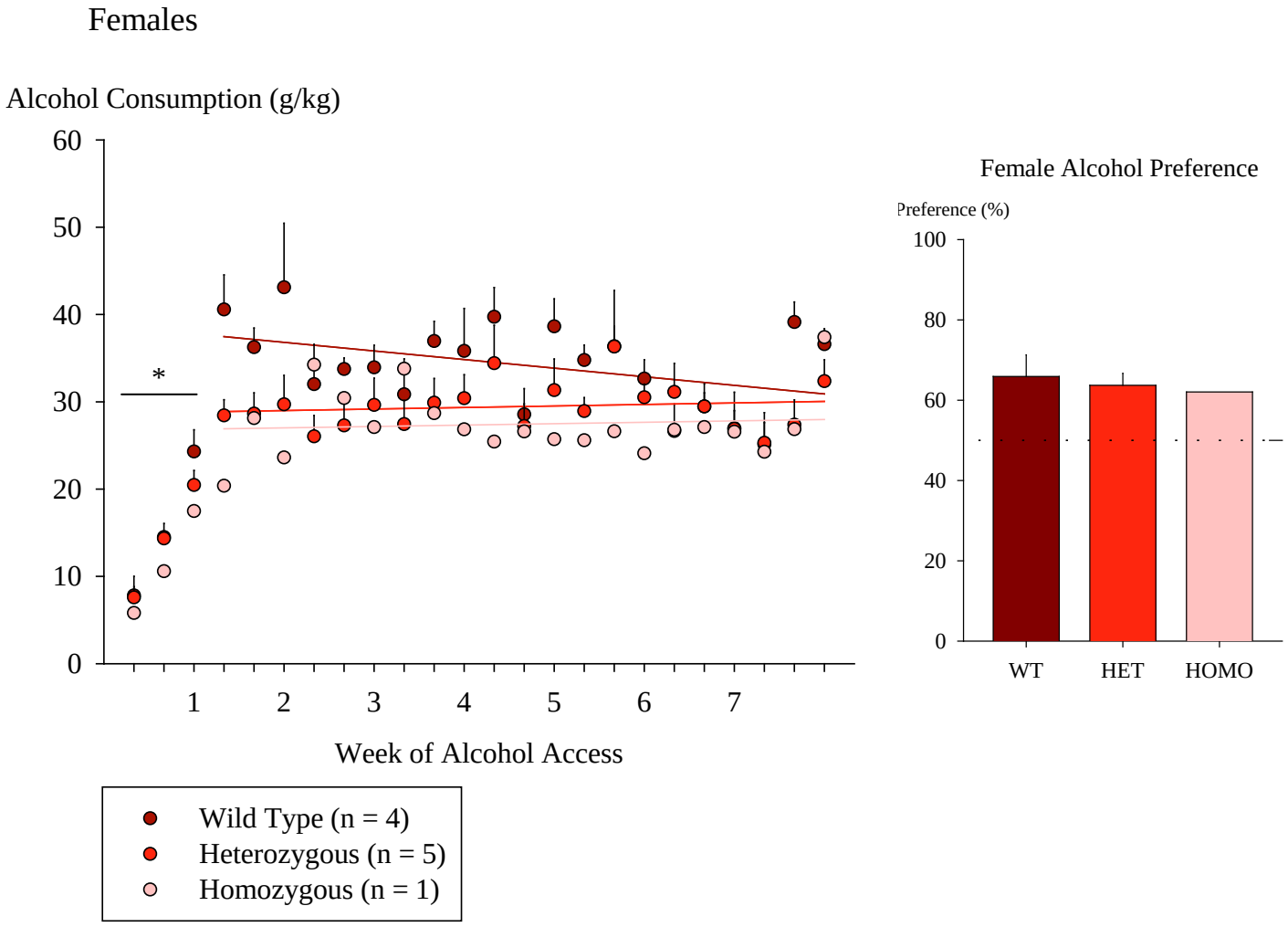
	Vehicle	0.5 mg/kg Allopregnanolone	10.0 mg/kg Allopregnanolone
Male Wild Type	7	6	8
Male Heterozygous	4	2	3
Male Homozygous	0	0	0
Female Wild Type	2	1	2
Female Heterozygous	2	2	1
Female Homozygous	0	0	0

Figure 1.



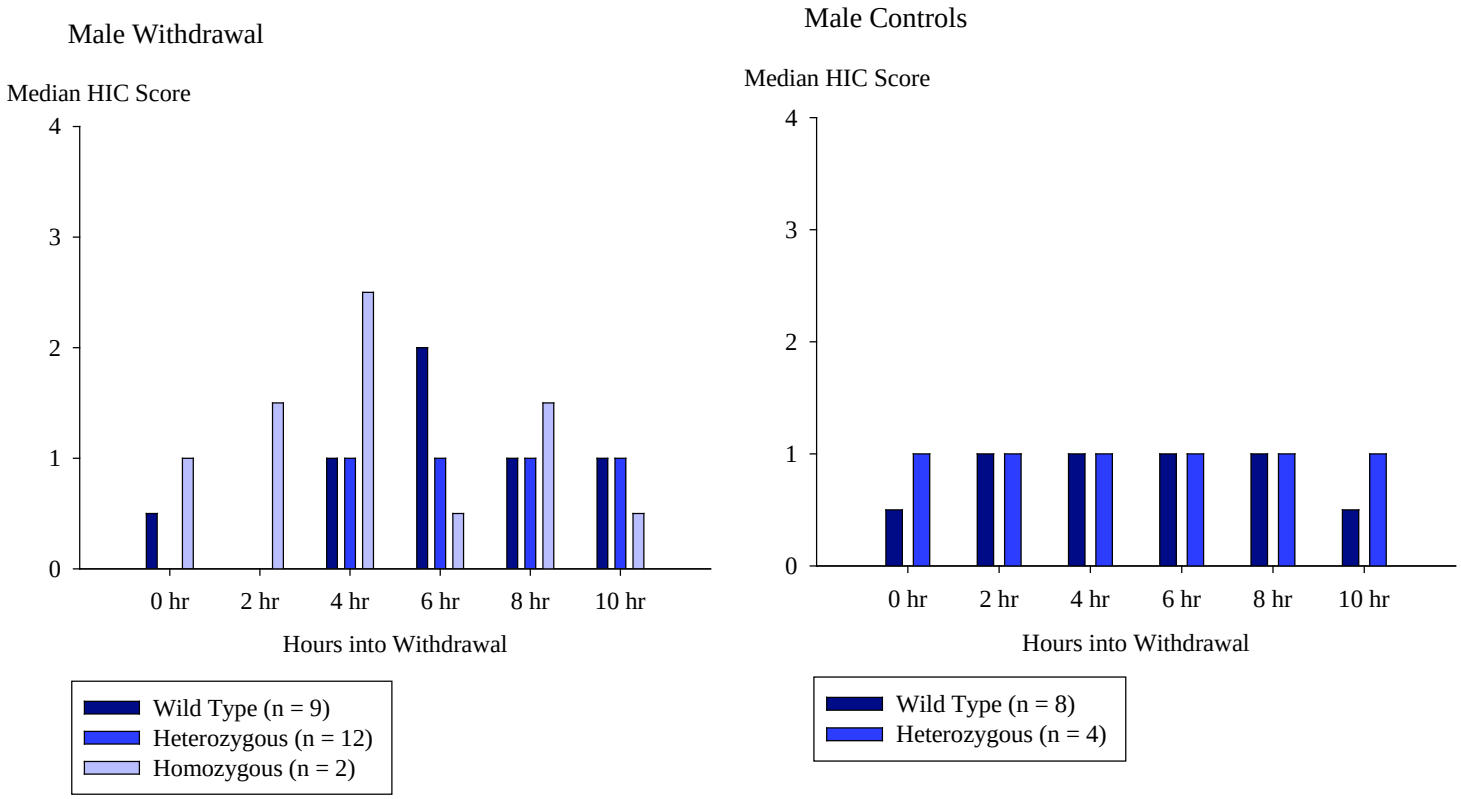
Alcohol consumption in the intermittent access protocol in male C57Bl/6 allopregnanolone insensitive mice showed stabilization over 8 weeks. There was no difference between genotypes for level of consumption or alcohol preference. There was a significant dose-effect for the 3, 6, and 10% alcohol fade in days ($p < 0.001$).

Figure 2.



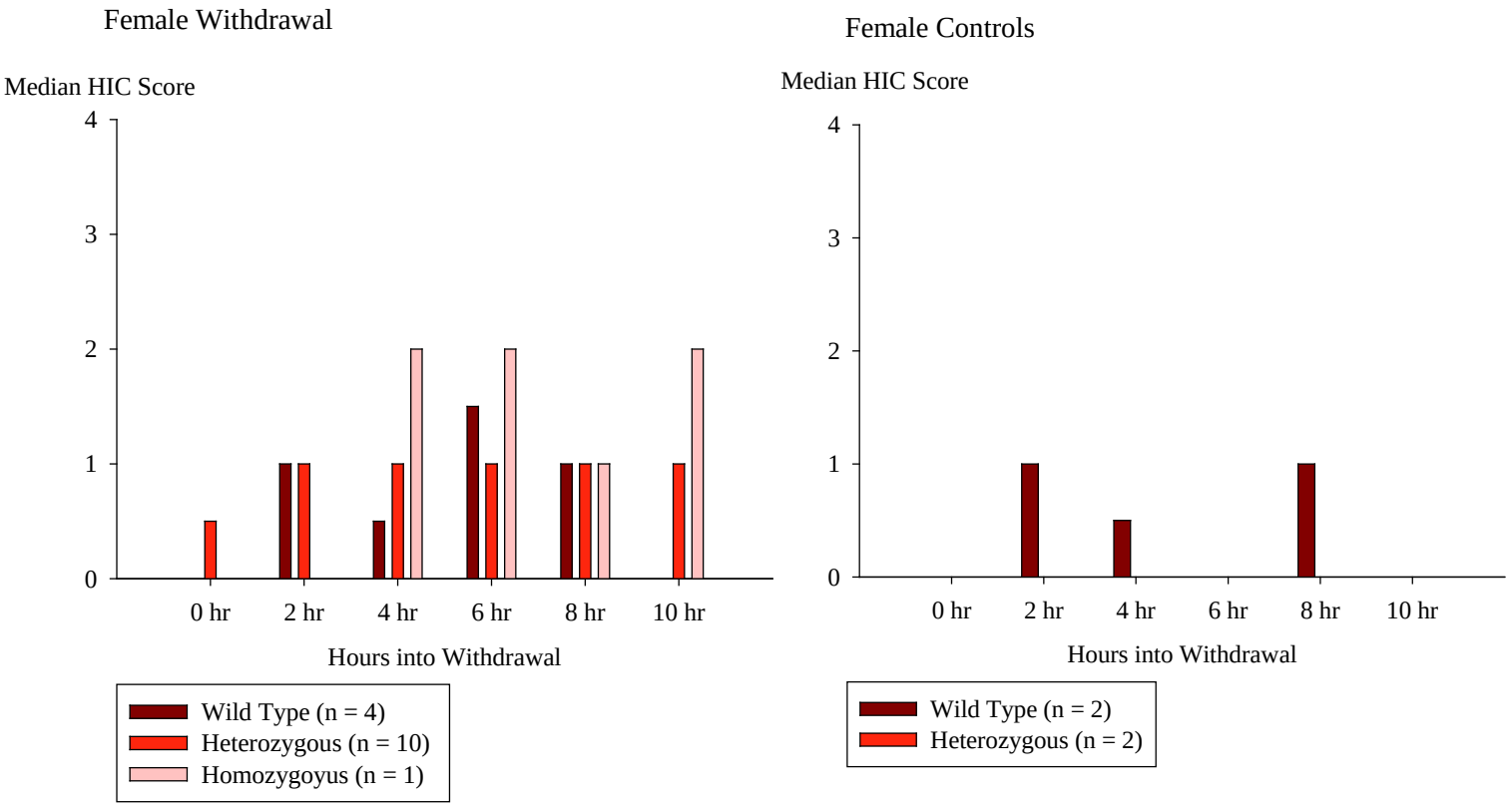
Alcohol consumption in the intermittent access protocol in femlae C57Bl/6 allopregnanolone insensitive mice showed stabilization over 8 weeks. Females consumed significantly more ethanol than males ($F(1, 36) = 70.84$, $p < 0.001$). There was no difference between genotypes for level of consumption or alcohol preference. There was a significant dose-effect for the alcohol fade-in days ($p < 0.001$).

Figure 3.



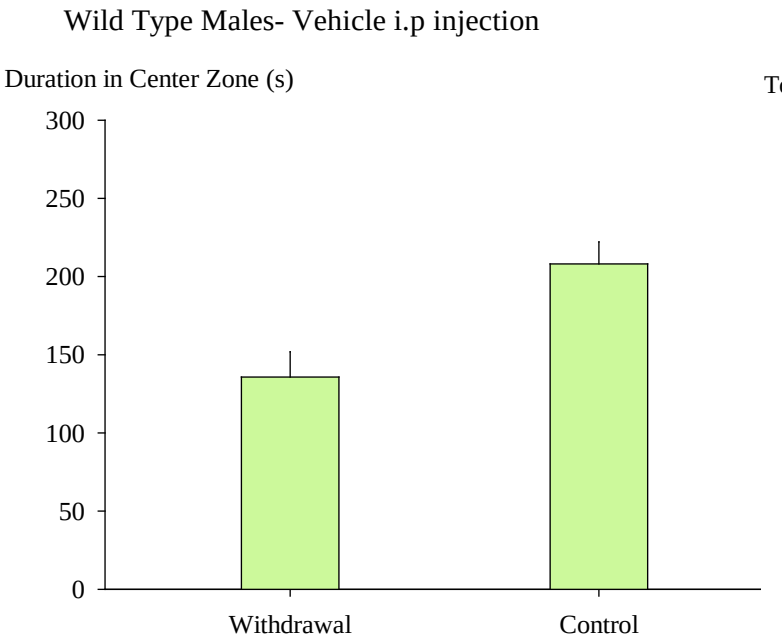
Handling-induced convulsion scores for male C57Bl/6 allopregnanolone insensitive mutants for animals in ethanol withdrawal and control animals. Males in withdrawal tend to show higher HIC scores than the control males. Homozygote males in withdrawal tend to show higher HIC scores over 10 hours.

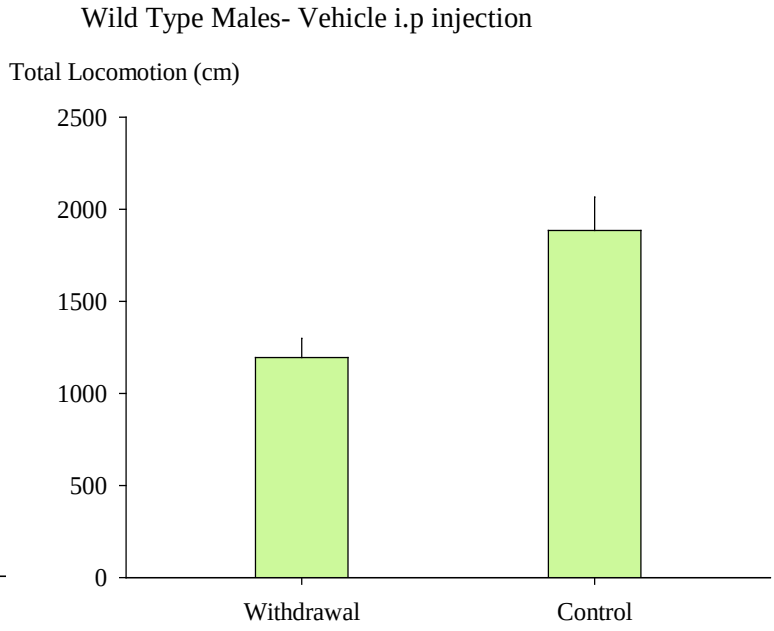
Figure 4.

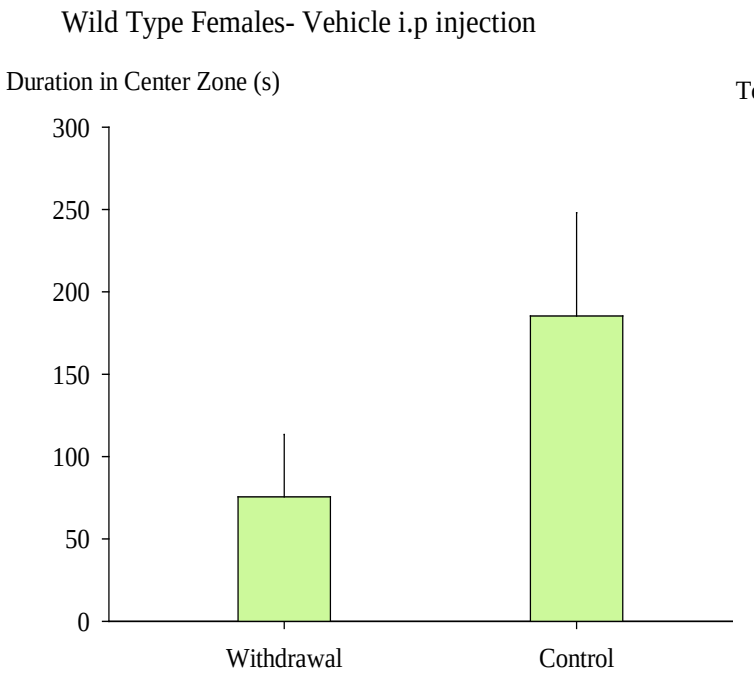


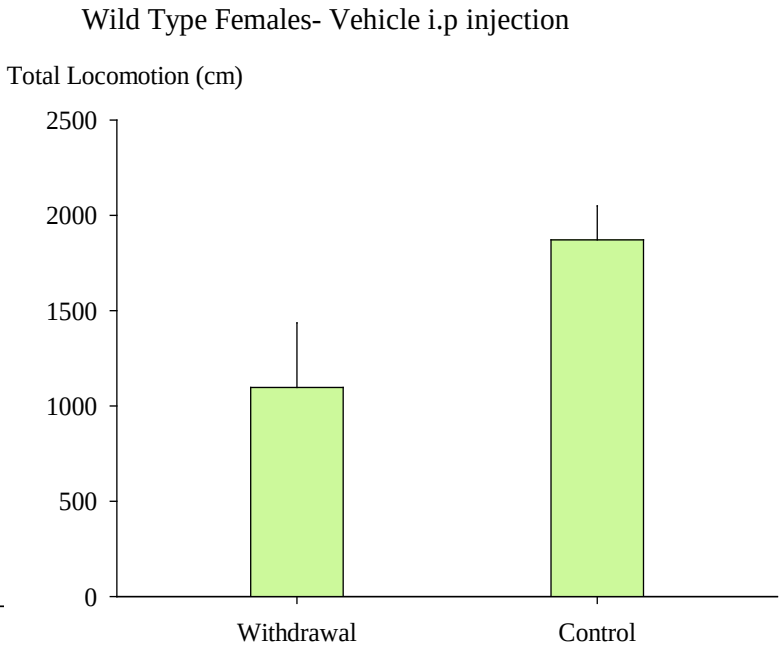
Handling-induced convulsion scores for female C57Bl/6 allopregnanolone insensitive mutants for animals in ethanol withdrawal and control animals. Withdrawal females tend to show higher HIC scores than control females. Homozygote females in withdrawal tend to show higher HIC scores over 10 hours into withdrawal.

Figure 5.



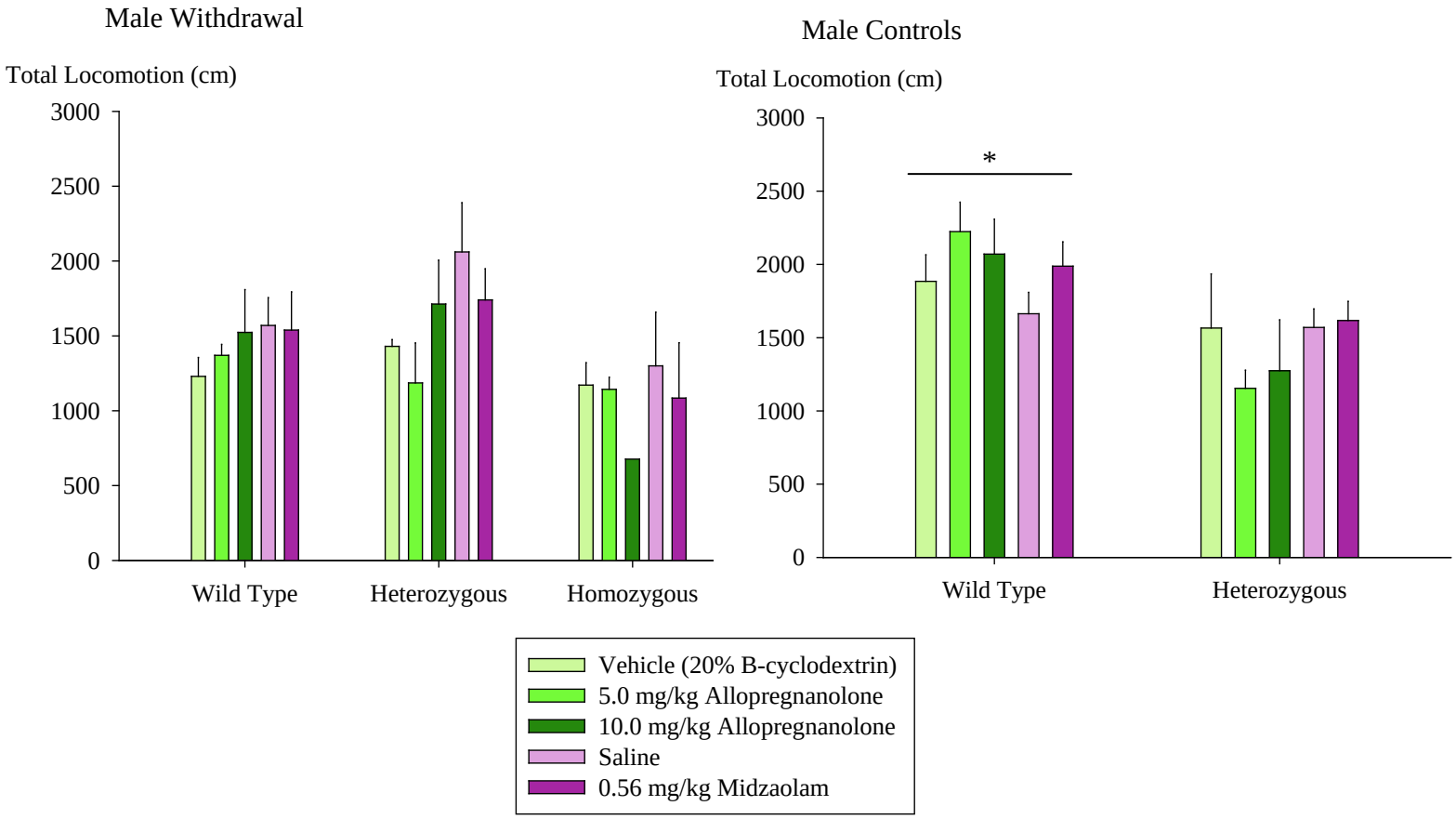






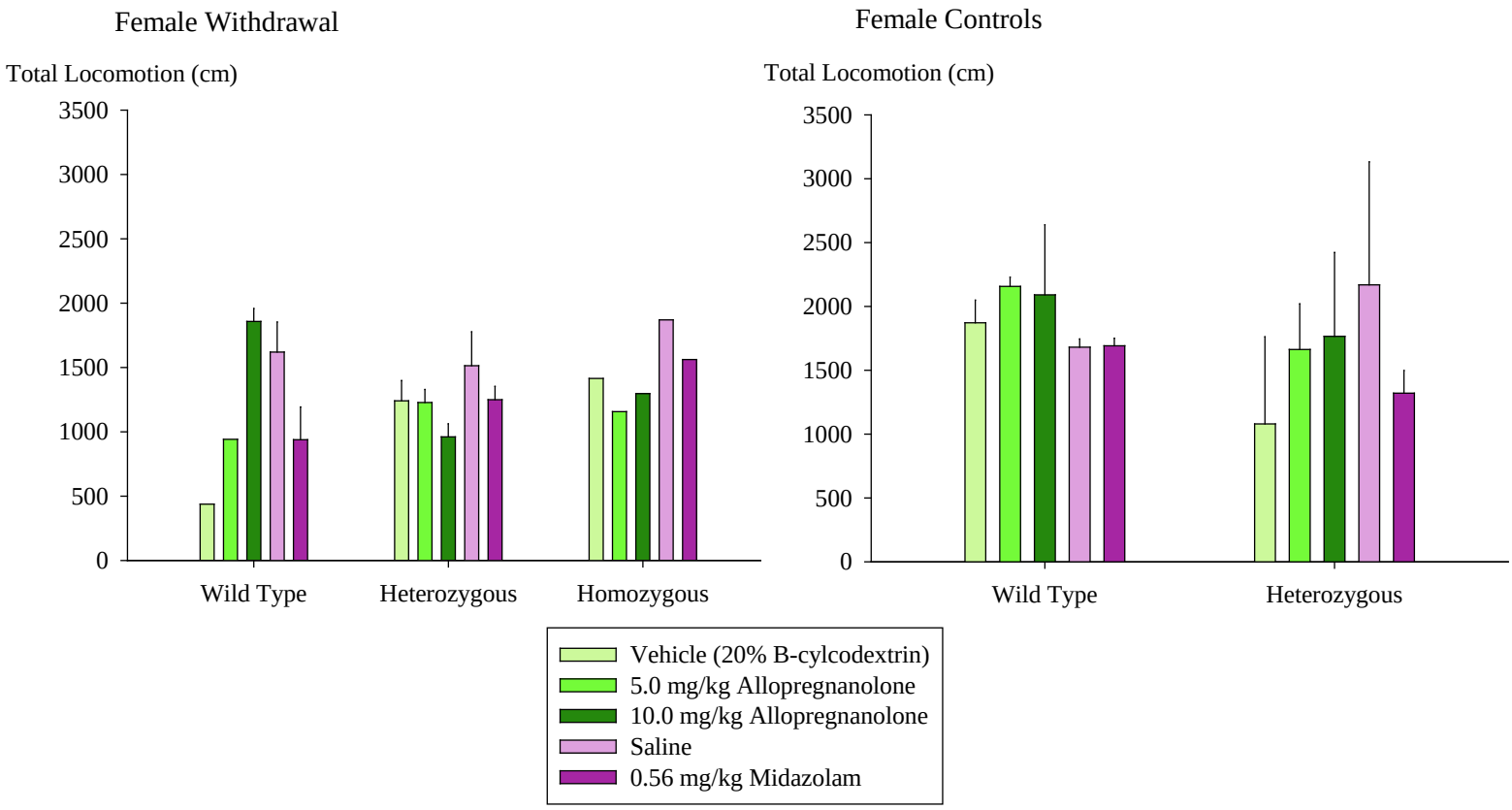
Male and female wild type animals in withdrawal from ethanol with the vehicle (20% B-cyclodextrin) drug treatment showed less time in the center zone and less total locomotion in the open field.

Figure 6.



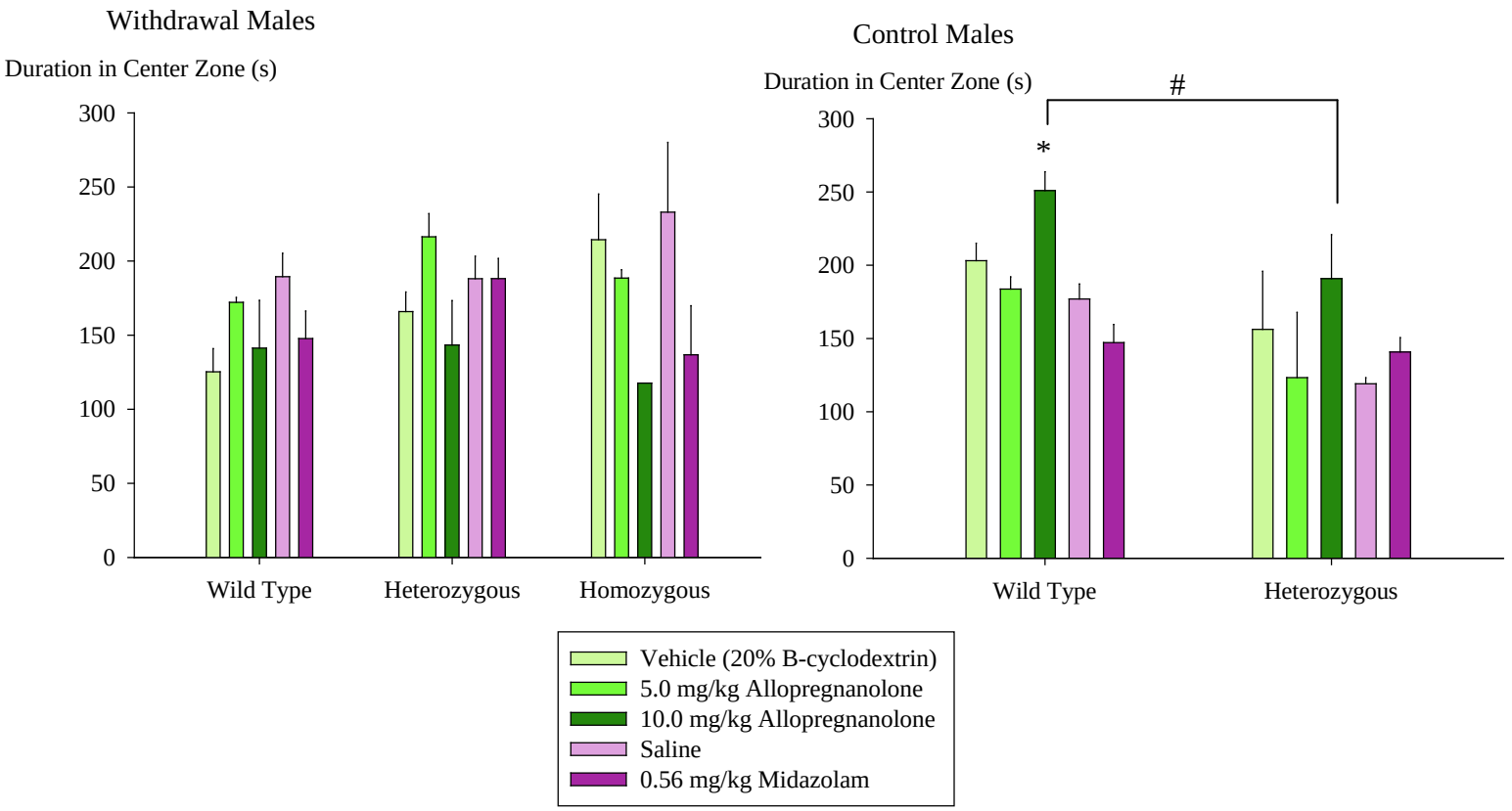
Male total locomotion for withdrawal animals and control animals in the first 5 minutes of the open field test. There is a significant difference in locomotion between wild type and heterozygous control males ($t = 3.16$, $p = 0.010$).

Figure 7.



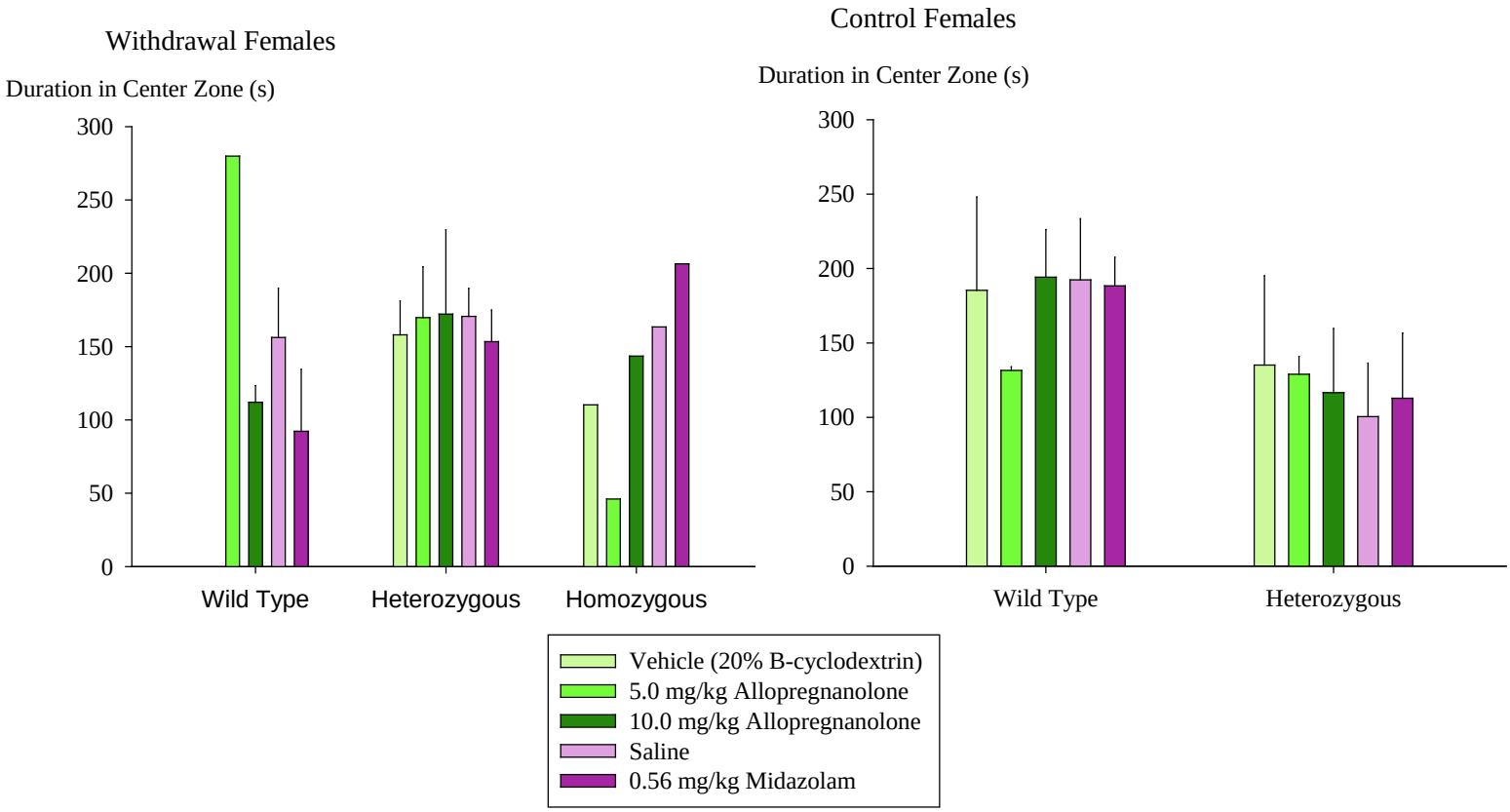
Female total locomotion for withdrawal animals and control animals in the first 5 minutes of the open field test.

Figure 8.



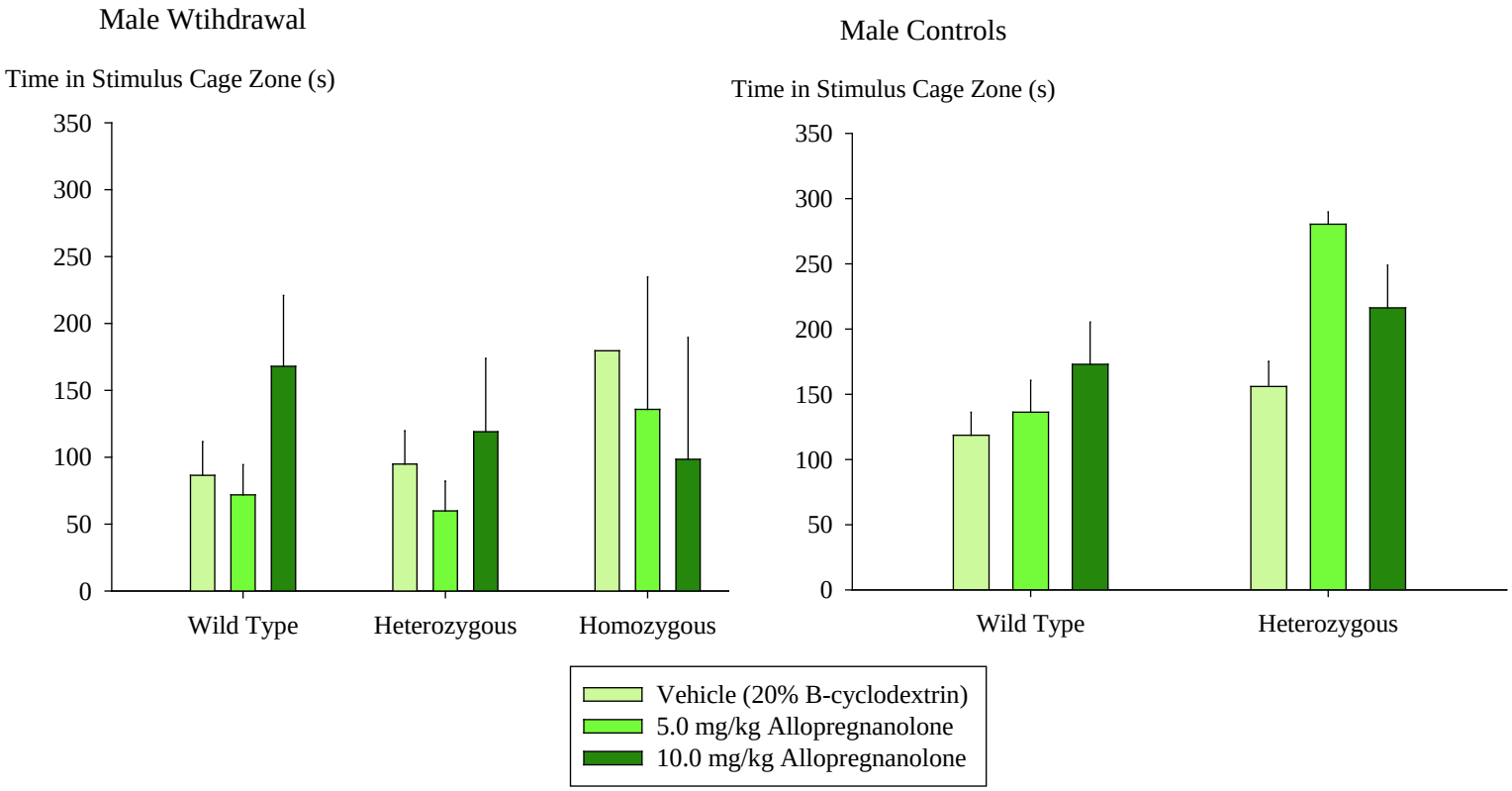
Male withdrawal and control mice: time in the center zone of the open field for the first 5 minutes of the 1-hour trial. For wild type and heterozygous control males, the 10.0 mg/kg dose of allopregnanlone increased time spent in the center zone versus the midazolam dose, the saline dose, and the 5.0 mg/kg dose of allopregnanolone ($t = 4.48$, $p < 0.001$; $t = 4.24$, $p = 0.001$; $t = 3.91$, $p = 0.003$, respectively). Within wild type males, the 10.0 mg/kg dose significantly increased time in the center zone over the midazolam, saline, and 5.0 mg/kg allopregnanolone dose ($t = 5.16$, $p < 0.001$; $t = 3.60$, $p = 0.009$; $t = 3.25$, $p = 0.023$, respectively).

Figure 9.



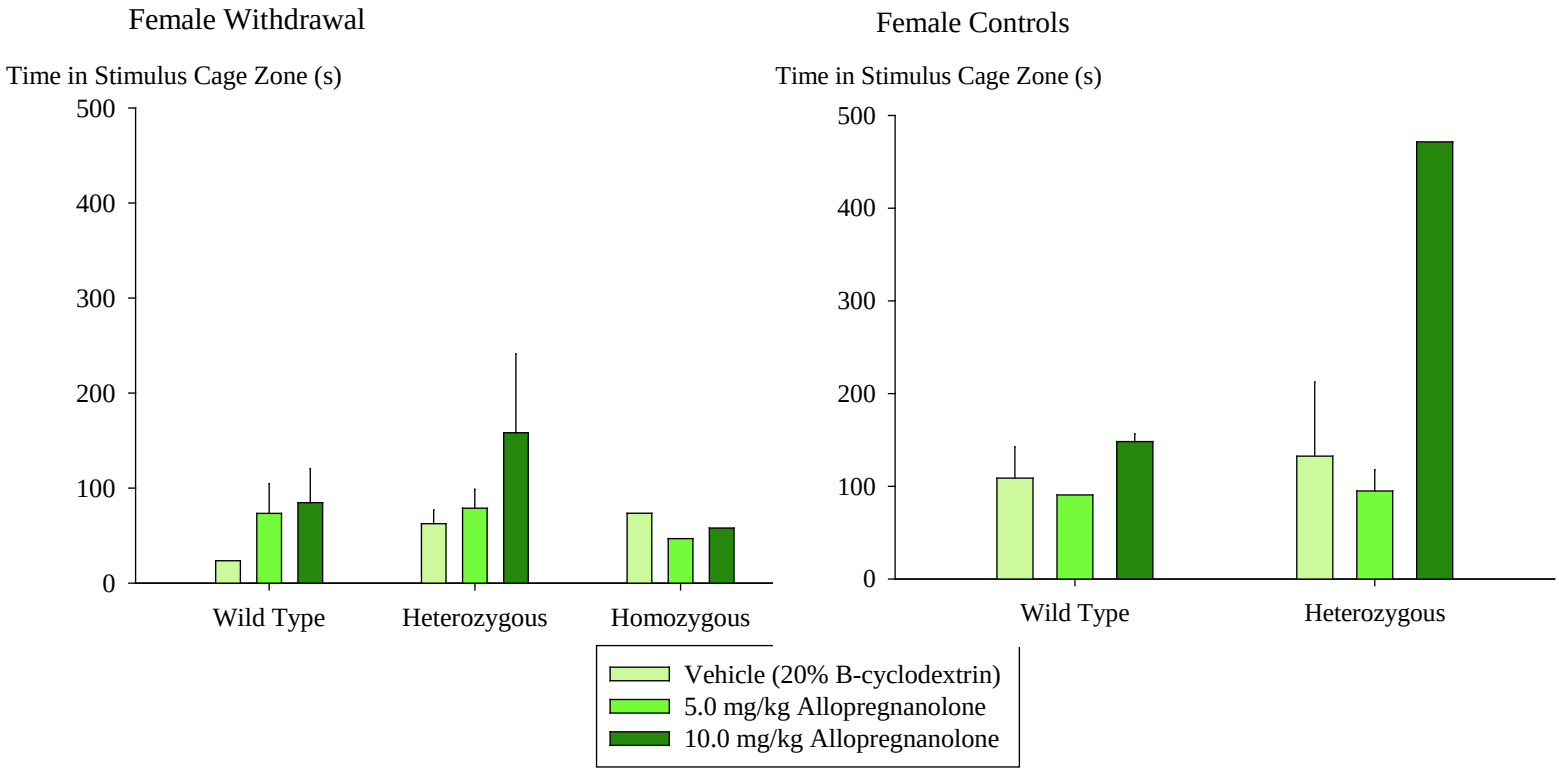
Female withdrawal and control mice time: time in the center zone of the open field for the first 5 minutes of the 1-hour trial. Females spend significantly less time in the open field than males ($p = 0.027$).

Figure 10.



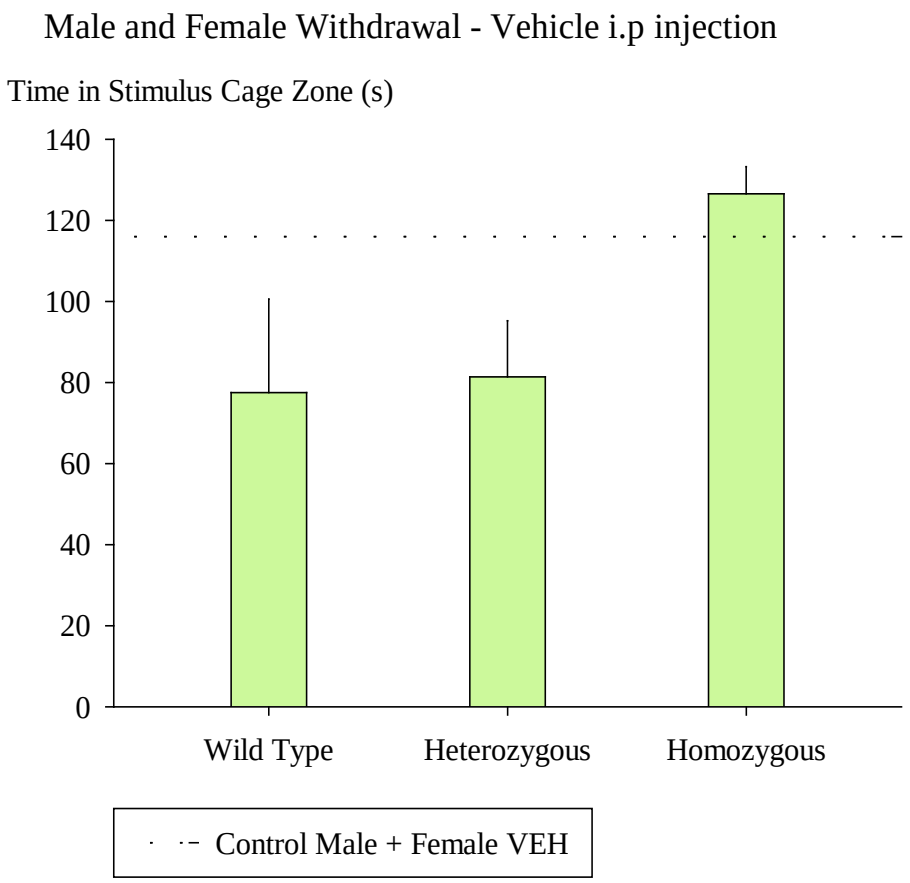
Male withdrawal and control time spent interacting with a novel OVX female CFW in the social preference test. The 5.0 mg/kg (t = 3.11, p = 0.023) and 10.0 mg/kg (t = 2.78, p = 0.044) significantly increased interaction time across genotypes for control males.

Figure 11.



Female withdrawal and control time spent interacting with a novel OVX female CFW in the social preference test.

Figure 12.



Male and female homozygous mice in withdrawal from ethanol had levels of interaction time that are comparable to male and female control animals with no ethanol history.

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