The Effects of Gene Knockout of the Vesicular Monoamine Transporter 2 (VMAT2; SLC18A2) and the Dopamine Transporter (DAT; SLC3A6) on Ethanol Consumption and Escalation in Mice.

Undergraduate Psychology Honor's Thesis

Alexandra Houston-Ludlam

Advisor: Dr. F. Scott Hall

Department of Psychology

University of Maryland, College Park

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INTRODUCTION

Alcoholism, including alcohol abuse and alcohol dependence, has a lifetime prevalence of 12-21% (Bronisch & Wittchen, 1992; Hasin, Stinson, Ogburn, & Grant, 2007; Latvala et al., 2009). The effects of alcoholism on our society can be measured in terms of healthcare costs, lost productivity and other deleterious effects including increased crime and automobile accidents that collectively cost the United States 185 billion dollars in 1998 (DHHS, 2000). A number of approaches for the treatment of alcoholism have been developed, including pharmacological treatments, although the success of these treatments remains minimal, at best. Thus, the development of more effective alcoholism treatments is an important societal goal, an endeavor that would be greatly accelerated by improved understanding of the factors that predispose individuals to alcoholism and of the underlying biological differences between alcoholics and non-alcoholics. To this end, while environmental and genetic factors are known to contribute to the development of alcoholism, the heritability of alcoholism is about 50% (Goldman, Oroszi, & Ducci, 2005). The nature of these genetic differences, the genetic structure of these effects, and the degree of genetic heterogeneity for those genetic differences is largely unknown, as are the psychological and behavioral processes that are specifically associated with these genetic differences. This last point may be particularly important as alcohol abuse frequently presents comorbidly with mood, personality, or anxiety disorders, suggesting that the mechanisms involved in these psychiatric disorders may overlap with those involved in alcoholism.

Effects of Alcohol in the Body

Ethanol is the alcohol present in typical alcoholic beverages. Ethanol is a small, uncharged polar molecule that moves freely across bilipid membranes by passive diffusion.

Thus, it is easily absorbed into the gastrointestinal tract, where it can readily diffuse into all biological tissues. Alcohol is metabolized in the liver by the enzyme alcohol dehydrogenase into acetaldehyde, which is further degraded into acetic acid by acetaldehyde dehydrogenase. These two enzymes are responsible for the metabolism of approximately 90% of orally consumed ethanol (Bullock, 1990). Acetic acid is further broken down into carbon dioxide and water in most tissues, including the pancreas, brain, and stomach (Zakhari, 2006).

As previously mentioned, the simple chemical structure of ethanol, compared to other drugs of abuse, allows it to penetrate cell membranes and the blood-brain barrier with ease. Alcohol has many specific and non-specific effects in the brain. The chemical structure of alcohols (the polar hydroxyl group combined with the carbon chain) allows alcohol molecules to interact with the phospholipid bi-layer that constitutes cell membranes, specifically by binding to hydrophobic areas of the membrane near transmembrane receptors. This affects the position of channel proteins and receptors, as well as local properties of the cell membrane. Specific actions of ethanol include actions at neurotransmitter binding sites, altering second-messenger production and stimulating release of several neurotransmitters through downstream actions (e.g. actions of ethanol at the serotonin 5-HT_3 receptor leads to release of dopamine; DA). Alcohol is able to interact with both G protein coupled receptors, by indirectly stimulating the G protein that activates the cAMP system, and ligand-gated receptors. γ-amino butyric acid (GABA) and glutamate, both acting through ligand-gated receptors, are affected by changes to their ligand-gated channels, while several serotonin (5-HT) receptors, dopamine (DA) receptors and $GABA_B$ receptors are affected by alcohol indirectly stimulating the function of G-protein-coupled receptors, thereby modifying signals sent via the cAMP second messenger system (Greengard, 2001; B. Johnson, 2004). As there are many G-protein coupled receptors involved in

neurotransmission, and they can have opposing actions, the effects of ethanol on specific neurotransmitter systems and specific neurotransmitter receptors are discussed in more detail in later sections. Even from this brief description, however, it is clear that many neurotransmitter systems mediate the effects of ethanol. Moreover, these neurotransmitter systems themselves, independently of the effects of ethanol, may be involved in ethanol consumption and self-administration by mediating behavior that influences ethanol consumption.

Dopamine

The DA system has been naturally thought to be involved in the rewarding effects of ethanol, given its primary role in motivation and reward (Schultz, 1997). However, an understanding of the dopaminergic system is important before evaluating specific relationships between DA signaling and ethanol consumption. As a catecholamine, DA has a catechol group and an amine group, and is a classical, small-molecule neurotransmitter synthesized in a two-step process. In the first step, the amino acid L-tyrosine is converted to L-DOPA by the enzyme tyrosine hydroxylase. L-DOPA is then converted into DA by amino acid decarboxylase. Conversion of tyrosine into L-DOPA is the rate-limiting step in DA synthesis (For review see Molinoff & Axelrod, 1971). Several drugs act on this process by either increasing precursor enzymes to increase DA production or antagonizing the synthesis enzymes to limit neurotransmitter synthesis (Elsworth & Roth, 1997).

After synthesis, DA is stored in synaptic vesicles until release. Transporters on the surface of neurotransmitter vesicles, primarily the vesicular monoamine transporter 2 (VMAT2) in the central nervous system, are responsible for moving DA into vesicles for storage and release. DA has five post-synaptic receptor subtypes, all of which are metabotropic. The first

DA receptors identified were the D_1 and D_2 subtypes, noted to have opposing actions (For review see Kebabian & Calne, 1979). Because of the similarity of the subsequent receptors identified (D_3, D_4 and D_5) to the D_1 and D_2 subtypes, these receptors were classified into subgroups as "D_1-like" or "D_2-like" receptors. D_1 like receptors include D_1 and D_5 receptors, and D_2-like receptors include the D_2, D_3, and D_4 subtypes (For review see Lachowicz & Sibley, 1997). This classification scheme is based upon nucleotide sequence similarity of the genes for these receptors, nucleotide sequence similarity of the mRNA for these receptors, amino acid sequence similarity of the receptor proteins, and structural similarity that leads to similar G-protein interactions for each receptor subclass and ligand interactions. Consequently, there is a similar pharmacology for early agonists and antagonists that could not discriminate between members of the two DA receptor subclasses. In many circumstances, the D_2-like receptors oppose the actions of the D_1-like receptors (although these are not always expressed on the same neurons). The primary action of the D_1-like receptors is to stimulate adenylyl cyclase, which increases levels of cAMP (Kebabian & Calne, 1979). Conversely, the D_2-like receptors inhibit adenylyl cyclase, therefore decreasing levels of cAMP (Werkman, Glennon, Wadman, & McCreary, 2006). There are two distinct variants of the D_2 receptor—the short version (D_{2S}) and long version (D_{2L}), created by alternative splicing during mRNA processing. DA release is modulated by D_2-like autoreceptors, e.g. D_{2S}, which reduce Ca^{++} entering the terminal, on the pre -synaptic membranes.

The dopamine transporter (DAT) is responsible for the reuptake of DA from the extracellular space to the cytosol. After reuptake, DA is transported back into vesicles by VMAT2 and can be used again for neurotransmission, although the majority of DA transported into vesicles is produced by *de novo* synthesis. If DA is not transported into the cytoplasm and

into vesicles, it is metabolized, which occurs through two main pathways. It is either broken down by monoamine oxidase A, located in catecholaminergic neurons, or monoamine oxidase B, located in astrocytes (Westlund, Denney, Kochersperger, Rose, & Abell, 1985), into 3,4-Dihydroxyphenol-acetic acid (DOPAC), which is then converted into homovanillic acid by catechol-O-methyl-transferase (COMT) in glia. Alternatively, DA is first metabolized by COMT into 3-methoxytyramine (3-MT) and further broken down by monoamine oxidase to produce HVA. DOPAC and HVA are then excreted into the CSF (For review see Kopin, 1985).

DA acts primarily as a neuromodulator by altering voltage sensitivity of neuronal membranes, as opposed to directly acting on ion channels to initiate action potentials (Di Chiara, 1995). DA acts primarily through D_1 and D_2 receptors located on the post-synaptic membrane. When DA is released and binds to D_1-like receptors, its G protein activates adenylyl cyclase, which is an enzyme responsible for cyclic adenosine monophosphate (cAMP) production. cAMP then associates with protein kinase A, which acts in the post-synaptic cell by inhibiting voltage gated potassium channels, G-protein regulated inwardly rectifying potassium channels, transient sodium channels, and certain subtypes of calcium channels. cAMP stimulates persistent sodium channels, NMDA receptors, AMPA receptors, and GABA receptors (For review see Neve, Seamans, & Trantham-Davidson, 2004). Conversely, association of DA with D_2-like receptors inhibits adenylyl cyclase, which inhibits signal transduction and alters permeability of potassium channels. Specific actions of reductions in cAMP include inhibition of calcium and sodium channels, and NMDA, AMPA, and GABA receptors. This also stimulates voltage gated and g-protein regulated inwardly rectifying potassium channels. This can cause increased membrane potential voltage, making membrane depolarization by subsequent action potentials less likely (For review see Neve et al., 2004). Actions of DA at D_2-

like autoreceptors causes inhibition of calcium influx following membrane depolarization. As calcium ions trigger vesicular fusion with the membrane, reducing cytosolic calcium levels reduces the amount of DA release. Actions of DA at potassium channels, which are necessary for action potential propagation, is an indirect mechanism of DA action that can greatly affect a nerve cell's ability to generate action potentials (For review see Girault J, 2004; Greengard, 2001).

The anatomy of the DA system was first characterized by Dahlström and Fuxe (1964), who identified three concentrations of DA-containing cell nuclei, which they termed the A8, A9, and A10 nuclei. These projections were recognized as the substantia nigra compacta (SNc; A9), the ventral tegmental area (VTA; A10), and the retrorubral area (A8), the latter making up part of the medial reticular formation (Berger, Gaspar, & Verney, 1991; Porrino & Goldman-Rakic, 1982; Williams & Goldman-Rakic, 1998). The SNc and VTA are the DA nuclei that send projections to the caudate/putamen (dorsal striatum in rodents), ventral striatum (which includes the nucleus accumbens; NAc), and frontal cortical areas (Nicola, Surmeier, & Malenka, 2000; For review see Schultz, 1999; For review see Wauquier, 1980).

With regard to dopaminergic contributions to the mechanisms of reward and addiction, the most frequently studied clusters of DA neurons are the SNc and the VTA, and their associated terminal fields. DA release in the NAc and striatum, innervated by DA projections from the VTA and SNc respectively, have been linked to the rewarding properties of virtually all classes of abused drugs, including ethanol (Cowen & Lawrence, 1999; Jones, Gainetdinov, Wightman, & Caron, 1998). Fadda, Argiolas, Melis, Serra, and Gessa (1980) showed that chronic treatment with ethanol increased tissue levels of DOPAC in the SNc as well as the caudate nucleus and frontal cortex, whereas acute treatment with ethanol produced an increase of

DOPAC in only the caudate nucleus. When evaluating these results, one must recognize that measuring tissue metabolites is an indirect index of neurotransmission. Using metabolite levels to infer levels of DA activity assumes a direct relationship between DA neurotransmission and metabolite concentrations, which may or may not be the case. Additionally, these levels are dependent on the time intervals between neurotransmission and when the metabolite levels are measured, amongst other factors. Finally, measuring tissue metabolite levels with *ex vivo* methods necessarily measures primarily intracellular levels of these metabolites. The development of the *in vivo* microdialysis technique allowed researchers to measure the levels of extracellular neurotransmitter levels directly by using a probe, with a semi-permeable membrane at the tip, inserted into a brain region of interest (Tossman, Jonsson, & Ungerstedt, 1986). Of importance for the present discussion, microdialysis allows the effects of local administration, peripheral injection, and self-administration of ethanol to be studied in freely moving animals (Yoshimoto et al., 1996). Indeed, microdialysis studies confirm that administration of ethanol increases levels of extracellular DA, in addition to other monoamines, in the NAc (Heidbreder & De Witte, 1993). The extent of DA release appears to determine some ethanol effects. Low baseline DA function, as measured through microdialysis, in inbred mice is associated with higher levels of ethanol consumption in a self-administration paradigm. These high levels of ethanol consumption can be reversed by increasing synaptic DA through treatment with L-DOPA concurrent with a dopa carboxylase inhibitor (carbidopa) or an MAO-B inhibitor (selegiline) (George et al., 1995). While microdialysis studies have provided a more direct method to evaluate DA release compared with previous analysis of DA metabolites alone, there is still some question as to whether increased levels observed with microdialysis are actually the result of increased DA cell firing, or more direct effects on DA release. Electrophysiological

methods, which compliment microdialysis analysis, have shown that ethanol increases DA cell firing in mesolimbic dopamine neurons (Diana, Pistis, Carboni, Gessa, & Rossetti, 1993).

With regard to post-synaptic DA function, antagonism of either D_1-like or D_2-like receptors reduces ethanol self-administration in rats, while low doses of the D_2-like agonist quinpirole alone increases self-administration. High doses of quinpirole alone reduce self-administration, but when quinpirole is administered with either D_1-like agonists or antagonists, increases in self-administration are observed. These results show a direct relationship of the dopaminergic receptors in mediating effects of ethanol action, and suggest an interaction between the D_1-like and D_2-like receptors on the effects of ethanol in the NAc (Hodge, Samson, & Chappelle, 1997). This evidence supports the idea that there is an important role of DA in ethanol consumption and reward. Any deficit or change to this system could in turn affect ethanol consumption. Biochemical and social environmental factors can affect DA system function and ethanol responses, and vice versa. Chronic treatment with ethanol can reduce baseline DA levels as measured by microdialysis in rats (Engleman, Keen, Tilford, Thielen, & Morzorati, 2011). Isolation rearing has been shown to change baseline DA function—rats reared in isolation showed elevated baseline DA levels as measured through microdialysis and a compensatory down regulation of D_2 receptors in the striatum (Hall, Wilkinson, et al., 1998). As such, it is not surprising that isolation rearing also increases ethanol consumption, particularly at higher concentrations of ethanol, regardless of baseline ethanol preference in rats (Hall, Huang, Fong, Pert, & Linnoila, 1998). Since chronic treatment with ethanol reduces baseline DA levels (Engleman et al., 2011), the effect of isolation rearing on ethanol consumption could model proposed “self-medication” in humans, where using ethanol or other substances of abuse normalizes some baseline deficit. Given these findings, it is clear that genetic variation that

affects neurotransmitter release dynamics and DA system signaling is quite likely to alter the effects of ethanol. Allelic variation in genes that code for transporters of DA in humans, and transgenic manipulations of these genes in animal models, might affect ethanol reward, reinforcement and consumption.

Serotonin

While DA certainly has a primary role in the reinforcing effects of ethanol, research also shows that 5-HT is involved in ethanol reinforcement and consumption, and indeed there is more evidence in humans for a role for 5-HT in alcoholism itself. However, this may involve the consequences of differences in 5-HT function on behavioral or psychological attributes that contribute to addiction, such as impulse control (Nielsen et al., 1994), rather than modulation of the effects of ethanol *per se*. 5-HT is implicated in many behaviors and neurological functions that may be important modulators of the effects of ethanol, ethanol consumption, and alcoholism, including anxiety, cognition, and mood regulation (For review see Lucki, 1998).

5-HT is synthesized from the amino acid tryptophan by the enzyme tryptophan hydroxylase to produce 5-hydroxy-L-trypotphan (5-HTP) (Renson, Goodwin, Weissbach, & Udenfriend, 1961). This is the rate-limiting step in 5-HT synthesis. 5-HTP is then converted into 5-HT by amino acid decarboxylase. 5-HT levels depend on the ratio of tryptophan to other neutral amino acids in the blood which compete for transport across the brain-blood barrier (Fitzpatrick, 1999), although, generally speaking, it requires some degree of intervention to induce circumstances in which central 5-HT function is affected by dietary tryptophan. After synthesis, cytosolic 5-HT is transported into vesicles for storage and release through vesicular membrane proteins like VMAT2. The removal of 5-HT from the synaptic cleft is mediated by

the serotonin transporter (SERT). 5-HT is metabolized by monoamine oxidase A into the metabolite 5-hydrosyindoleacetic acid (5-HIAA), which can be measured in samples of CSF, as an indicator of serotonergic activity (For review see Hannon & Hoyer, 2008)

Serotonergic cell nuclei are found throughout the midbrain, pons, and medulla, projecting widely throughout much of the forebrain, as well as to other structures, including the spinal cord. The most highly concentrated area of 5-HT-containing neurons is along the midline of the brain stem (e.g. raphé nuclei from the Greek word for "seam") where nine 5-HT-containing cell groups were originally described, B1-B9 (Dahlström & Fuxe, 1964). The B6/B7 groups constitute the dorsal raphé nucleus and the B8 group is the median raphé nucleus. The dorsal and median raphé nuclei send projections widely to most areas of the forebrain and neocortex, including many parts of the limbic system, and are consequently involved in a wide range of functions relevant to the effects of ethanol.

The 5-HT receptor family is quite large, and includes seven types of receptors, most of which have several subtypes (For review see Hannon & Hoyer, 2008). The 5-HT_1 receptor subfamily is the largest with five subtypes—5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, and 5-HT_{1F}. The 5-HT_1 receptors are G-protein linked receptors that cause a decrease in production of cAMP when activated. The two main 5-HT_1 receptors involved in the actions of ethanol are 5-HT_{1A} and 5-HT_{1B}. 5-HT_{1A} receptors are mainly inhibitory somatodendritic autoreceptors, acting by inhibiting adenylyl cyclase and increasing activity of potassium channels, the latter causing membrane hyperpolarization. 5-HT_{1B} receptors also serve as autoreceptors, and are located on the post-synaptic cell membrane as release modulating heteroreceptors. The 5-HT_2 receptor subfamily contains three members, 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C}, and this receptor family is directly involved in the effects of ethanol. 5-HT_{2A} receptors are known to act through the

phosphoinositide second messenger system, increasing intracellular calcium levels, which is a signal for vesicular fusion with the cell membrane. The 5-HT_{2B} receptors act as presynaptic inhibitors, and work in conjunction with the serotonin transporter to regulate serotonin release (Callebert et al., 2006). Activation of the 5-HT_{2C} receptor inhibits DA and norepinephrine release (Alex, Yavanian, McFarlane, Pluto, & Pehek, 2005), one of the diverse interactions between the 5-HT and DA systems. 5-HT_3 receptors cause rapid membrane depolarization, by opening non-selective cation channels, one consequence of which is to stimulate DA release by presynaptic facilitation. There are four more subtypes of 5-HT receptors that are not known to have distinct roles in mediating the effects of ethanol, but are less well understood. 5-HT_4 receptors are excitatory in nature, and promote cAMP production through activation of adenylyl cyclase. 5-HT_5 receptors are thought to act in a similar manner to 5-HT_1. Lastly, both 5-HT_6 and 5-HT_7 receptors act through G-protein coupled mechanisms to increase cAMP production (For review see Hannon & Hoyer, 2008; Hayes & Greenshaw, 2011; For review see Hoyer, Hannon, & Martin, 2002). As mentioned, the receptors that have been most frequently shown to be involved with ethanol consumption are the 5-HT_1, 5-HT_2, and 5-HT_3 and receptors. These receptors are found throughout the cortex, and in areas associated with DA reward—SNc, VTA, NAc, and caudate-putamen. .

There is substantial evidence that the 5-HT system is involved in alcoholism in humans (For review see LeMarquand, Pihl, & Benkelfat, 1994), as well as in the effects of ethanol based on studies in animals. In some samples of alcoholic patients, low levels of the 5-HT metabolite 5-HIAA in cerebrospinal fluid samples were observed (Linnoila et al., 1983). Reduced 5-HIAA levels have also been associated with increased ethanol consumption in rhesus monkeys (Higley, Suomi, & Linnoila, 1996). Individuals with a family history of alcoholism have been reported to

have an increased rate of uptake of 5-HT, effectively reducing the extracellular levels of 5-HT available to interact with 5-HT receptors (Rausch, Monteiro, & Schuckit, 1991). However, reduced levels of serotonin transporters were reported in a sample of alcoholics (Heinz et al., 1998), and more recent studies have indicated that there is a reduction of available serotonin transporters in alcoholic patients (Ho et al., 2011). Those authors suggested a connection between polymorphisms in the promoter for the serotonin transporter and anxiety, depressive, and alcohol abuse symptomology. In a clinical sample of in-patient recovering alcoholics, 5-HIAA levels were significantly lower than controls and recently admitted patients with an alcohol abuse diagnosis, suggesting that serotonergic neurotransmission is reduced following extended (1-2 months) abstinence from alcohol (Ballenger, Goodwin, Major, & Brown, 1979). This last finding in particular becomes hard to interpret—this effect could be due to chronic ethanol consumption, ethanol withdrawal, a pre-existing deficit in serotonergic neurotransmission, or some interaction between two or more of these factors—a general problem in human clinical studies.

Research in animals allows manipulation of 5-HT systems not possible in humans that may help to explain the involvement of the 5-HT system in the effects of ethanol and alcoholism. Low endogenous 5-HT levels in rats are linked with higher ethanol preference and consumption, and blocking 5-HT uptake reduces voluntary ethanol consumption (Daoust et al., 1985). Fluoxetine, a selective serotonin reuptake inhibitor, decreases intravenous self-administration of ethanol, consistent with 5-HT having an direct role in mediating effects of ethanol (Lyness & Smith, 1992), or perhaps for low 5-HT levels in alcoholism. Ethanol administration causes significant increases in extracellular 5-HIAA, the primary metabolite of 5-HT, (Heidbreder & De Witte, 1993) and further research with *in vivo* microdialysis showed increases in extracellular 5-

HT itself (Yan, Reith, Jobe, & Dailey, 1996). Further investigation by Yoshimoto and colleagues (1996) using *in vivo* microdialysis has shown that chronic alcohol consumption desensitizes the 5-HT system in the NAc. Specifically, alcohol increases 5-HT release onto 5-HT_3 receptors, which increases DA release in the NAc. Administering 5-HT_3 antagonists reduces DA release produced by alcohol, thereby reducing alcohol consumption as well (For review see LeMarquand et al., 1994b).

Glutamate, GABA and other actions of ethanol in the CNS

While ethanol consumption and alcoholism involve DA and 5-HT systems, there are other well-known effects of ethanol on other neurotransmitter systems, which might also contribute to the behavioral effects of ethanol. Ethanol affects glutamate, the primary excitatory neurotransmitter in the brain (Hayashi, 1952), by binding to the N-methyl-D-aspartate (NMDA) receptor, a ligand-gated cation channel responsible for membrane depolarization (Woodward, 1999). Membrane depolarization is mainly mediated by AMPA receptors; however, one must consider the synaptic plasticity of the CNS and realize that multiple receptors are able to cause similar effects, as is the case with NMDA and AMPA receptors. With acute treatment, ethanol inhibits the NMDA receptor, reducing glutamatergic neurotransmission (Lovinger, White, & Weight, 1989). Glutamate is well known to be involved in memory and learning, so it follows that NMDA receptor inhibition constitutes the underlying mechanism for memory loss associated with high levels of acute ethanol intoxication (Diamond & Gordon, 1997). One response to chronic ethanol consumption is an increase in the expression of NMDA receptors in an attempt to balance the reduced glutamate activity caused by alcohol (Qiang, Denny, & Ticku, 2007), and withdrawal symptoms of hyper-excitability have been associated with increased

glutamate transmission, measured by microdialysis, following termination of repeated alcohol consumption in rats (Fadda & Rossetti, 1998). Excess release of glutamate is damaging to nerve cells, so the increased glutamate neurotransmission caused by ethanol during withdrawal is also thought to contribute to brain damage seen following ethanol consumption, especially following repeated, intermittent and excessive consumption (See Tsai & Coyle, 1998 for review; Tsai et al., 1998).

GABA is well known to be the primary inhibitory neurotransmitter in the brain (McCormick, 1989). It follows that GABA has been shown to be responsible for many of the sedative effects seen with consumption of ethanol. $GABA_A$, a fast acting ligand-gated ion channel, binds GABA in the extracellular space and mediates an influx of Cl- into the cell, causing hyperpolarization. Ethanol acts at $GABA_A$ by enhancing Cl- conductance via the receptor (For review see(Grant, 1994)) and thus produces many effects similar to other indirect $GABA_A$ agonists, such as benzodiazepines. Treatment with the $GABA_A$ antagonist bicuculline attenuates Cl- currents produced by $GABA_A$ receptors (Suzdak, Schwartz, Skolnick, & Paul, 1986). However, some groups of neurons containing $GABA_A$ receptors show no changes in these potentials when treated with ethanol (Celentano, Gibbs, & Farb, 1988) and cultured cells show quite varied responses to ethanol, with only some cells showing an increase in Cl- conductance (Aguayo, 1990). This would suggest that there is a functional difference in $GABA_A$ receptor subunit composition that determines responsiveness to ethanol. Electrophysiological studies show different $GABA_A$ receptor sensitivities to ethanol in different brain regions (Peris, Coleman-Hardee, Burry, & Pecins-Thompson, 1992). Specific subunits of the $GABA_A$ receptor appear to have a more intense response to ethanol, including the $\alpha 1$, $\alpha 6$, $\beta 2$ and $\gamma 2$ subunits (for review see Loh & Ball, 2000), although the evidence is not yet conclusive (for review see Korpi

et al., 2007). Nonetheless, reduction of GABA neurotransmission, through treatment with antagonists, has been shown to reduce observable signs of ethanol intoxication and anxiolytic function following ethanol consumption (Grobin, Matthews, Devaud, & Morrow, 1998). The mechanism of ethanol's interaction via the $GABA_A$ receptor has been further corroborated by genetic models producing increased ethanol intoxication in mice, including long-sleep mice and mice with reduction of protein kinase C. Long-sleep mice have an increased latency to regain loss of righting reflex following acute ethanol injections, and elevated ethanol induced Cl- uptake. Other proteins involved in $GABA_A$ action, such as protein kinase C (responsible for phosphorylation of the GABA-A receptor subunits), have been investigated for their involvement in the effects of ethanol. Mice with reduced levels of a protein kinase C subtype also showed reduced behavioral changes following treatment with ethanol, and these same mice showed no enhanced Cl- conductance via GABA mediated mechanisms (For review see Mihic & Harris, 1997).

Finally, ethanol also interacts with the opioid system by increasing endogenous opioid (endorphin and enkephalin) release with acute ethanol consumption and by decreasing endorphin levels with chronic ethanol consumption (For review see Gianoulakis, 1989). There is opioid dependent and independent DA release following ethanol treatment. The primary areas in which opioids mediate DA release related to ethanol include the VTA and NAc (Gonzales & Weiss, 1998; Herz, 1997). The role of the opioid system in the actions of ethanol has been further supported by research with antagonists of opioid receptors, particularly the μ and δ opioid receptors (For review see Froehlich, 1997). Treatment with opioid receptor antagonists reduces ethanol self-administration in animals (For review see Ulm, Volpicelli, & Volpicelli, 1995). Research with knockout mice has shown that deletion of the μ opioid receptor largely eliminates

ethanol self-administration (Roberts et al., 2000) and other rewarding effects of ethanol (Hall, Sora, & Uhl, 2001).

While ethanol has actions on multiple neurotransmitter systems, it is important to remember that these systems are not isolated from one another, and that these neurotransmitters are part of an interacting neural circuit underlying ethanol reinforcement in which DA neurons form a central role. For example, endogenous opioid release modulates DA release in the NAc and VTA (Herz, 1997). It is clear that ethanol has widespread effects throughout the brain. These effects are associated with a number of brain systems and ethanol's actions on neurotransmitter systems are complimentary and multifaceted. A prime example of this is the interactions between DA, 5-HT, and their respective transporters. Indeed, there is a clear interaction of 5-HT at DAT—DAT will transport 5-HT in the absence of the serotonin transporter (Stamford, Kruk, & Millar, 1990; Zhou, Lesch, & Murphy, 2002). Previous research supports a role of 5-HT_3 receptors mediating the reinforcing and stimulating effects of ethanol via DA neurotransmission. A 5-HT_3 receptor antagonist reduces the extracellular DA increase following ethanol treatment (Wozniak, Pert, & Linnoila, 1990). There is clearly a role for 5-HT_3 receptors in regulating DA release following ethanol consumption (A. D. Campbell, Kohl, & McBride, 1996). The stimulating effects of ethanol on DA neurons may be caused by the inhibition of GABAergic mediated disinhibition of the DA neurons and a subsequent increase in dopaminergic neurotransmission (Mereu & Gessa, 1985). Changes to one system can have widespread effects on the entire response to ethanol in the system. Therefore, transporters or receptors that are able to act on multiple neurotransmitters, such as DAT and VMAT2, may affect an individual's response to ethanol and also act as targets for treatments of alcohol related disorders. Understanding the role of DAT and VMAT2 is important in determining the effects of

these transporters on ethanol in the brain, and the potential roles for these transporters in the etiology of alcohol related disorders.

Role of DAT and VMAT2 in determining monoamine sequestration

There are a number of direct links between DA and 5-HT systems involved in the reinforcing effects of ethanol. One important mediator of DA effects in the brain that might affect responses to ethanol is DAT (G. R. Uhl, 1992), which is a 12 segment transmembrane protein located in the pre-synaptic plasma membrane of DA neurons that is an important regulator of synaptic DA concentrations. This transporter mediates reuptake of released DA into presynaptic terminals, and is the primary transporter involved in removing extracellular DA from the extracellular space and moving it back to the cytosol in most brain regions (Mateo, Budygin, John, Banks, & Jones, 2004; G. R. Uhl, 1992). As an oddity of DA function, the primary mediator of DA uptake in the frontal cortex is NET (Moron, Brockington, Wise, Rocha, & Hope, 2002), which is consistent with the generally low levels of DAT expression in this region. In any case, in most DA terminal regions DA is co-transported into the cytosol from the extracellular synaptic space through DAT, which couples transport of DA with transport of Na+/Cl- ions down their respective concentration gradients (Torres, Gainetdinov, & Caron, 2003).

Some drugs of abuse act directly via DAT by causing a conformational change in the transporter such that it is unable to transport DA to the cytoplasm, e.g. transporter inhibition (Chen, Ferrer, Javitch, & Justice, 2000), or by reversing the action of DAT producing non-exocytotic DA release (Jones, Gainetdinov, Wightman, et al., 1998). Both mechanisms increase extracellular DA, but in manners that interact differentially with levels of DA cell firing. Current research supports that ethanol causes increased DAT expression on the surface of neuronal

membranes (Mayfield, Maiya, Keller, & Zahniser, 2001). Ethanol leads to higher levels of extracellular DA in areas of the brain associated with reward/reinforcement of ethanol consumption and ethanol self-administration (Stamford et al., 1990; Zhou et al., 2002), but through other mechanisms.

Another important regulator of cytosolic, and consequently extracellular, levels of DA and other monoamines is VMAT2. VMAT2 is one of two 12 transmembrane domain proteins involved in packaging monoamine neurotransmitter molecules into vesicles for sequestration, transport, and synaptic release (For review see Eiden, Schäfer, Weihe, & Schütz, 2004; Eiden & Weihe, 2011). VMAT2 mediates transport of the monoamines DA, 5-HT, norepinephrine and histamine into synaptic vesicles. This includes both monoamines brought back into the cytoplasm after secretion through transporters such as DAT, and newly synthesized monoamines (Fon et al., 1997). Transport via VMAT2 is coupled to translocation of protons, with a proton pump maintaining high intravesicular pH levels (Parsons, 2000). The evolutionary precursors to VMAT2 and other neurotransmitter transporters seem to have been involved in isolation and transport of toxins out of the cytosol. VMAT2 serves in a similar capacity in that it can transport toxic, or potentially toxic, chemicals (e.g. the monoamines themselves) out of the cytosol into the vesicle for transport elsewhere (Chaudhry, Edwards, & Fonnum, 2008; Guillot & Miller, 2009). Antagonists of VMAT2, including reserpine and amphetamine-like compounds, are thought to act by non-competitively binding to a site near the monoamine binding site of VMAT2, effectively blocking the active site and increasing cytosolic neurotransmitter levels (Partilla et al., 2006; Yasumoto et al., 2009). One of the consequences of such inhibition can be the cytosolic accumulation of toxic metabolites of the monoamine neurotransmitters as increasing levels overwhelm normal metabolic capacity. The “loading” of the neurotransmitter vesicles

also determines, in part, the amount of neurotransmitter available for release.

Thus, DAT and VMAT2 work in a concerted fashion to recycle DA and 5-HT from the extracellular space, specifically from the synaptic cleft back into the pre-synaptic cell and into synaptic vesicles. DAT mediates the re-uptake of DA from the extracellular space into the cytosol, and VMAT2 can transport the free DA and 5-HT from the cytosol into vesicles. These vesicles are then used to transport DA and 5-HT elsewhere in the synapse or for release into the synaptic cleft, and also to prevent a large amount of DA or 5-HT accumulating in the cytosol, which can have toxic effects on the cell. As suggested previously, since these transporters impact multiple neurotransmitter systems, and ethanol has widespread effects in the brain, changes occurring to dopaminergic and serotonergic function via DAT or VMAT2 could influence the development of alcohol related disorders. Even if these specific transporters are not directly involved in alcoholism, it could also be that manipulation of these transporters, both genetically and pharmacologically, could serve as a route to treat alcoholism. The advantage of animal research in this situation is that researchers are able to manipulate genes actively in order to identify which genes might have a role in given disorder. Research into alcohol related disorders often include genetic and behavioral animal models to explore effects of gene manipulation on ethanol related behavior.

Genetic Factors and Alcohol Consumption

Genetic models of alcohol dependence are often used in combination with behavioral models and pharmacological manipulations, to provide important information regarding the role genetics plays in alcohol consumption and alcohol dependence. The most common genetic models used in research of alcohol dependence involve selectively breeding animals who show

aversion to, or preference for, alcohol, and by selectively modifying specific gene sequences using transgenic techniques in order to eliminate or alter the function or expression of specific genes. In some cases mice with certain traits, such as high or low acute functional tolerance to ethanol (Erwin & Deitrich, 1996) or high ethanol consumption (Crabbe, Spence, Brown, & Metten, 2011), are inbred to produce offspring with the same trait, with divergence of the trait occurring over just a few generations. This shows that the traits are themselves heritable, although it does not identify the specific genes involved. Comparison between these strains may lead to identification of the relevant genes, although other techniques may be more amenable to this goal. As discussed above, the effects of alcohol and alcoholism are thought to involve many brain systems and consistent with this assertion substantial genetic and neurobiological evidence has implicated both DA and 5-HT systems in the effects of ethanol and in alcoholism (Ducci & Goldman, 2008). Specifically, candidate gene studies have implicated both the DAT (Dobashi, Inada, & Hadano, 1997; Ueno et al., 1999) and the VMAT2 (Lin et al., 2005) genes in alcoholism.

Genetic Factors in Alcoholism

Twin Studies

Family and twin studies have been informative in determining the amount of genetic versus environmental influence to developing alcoholism. In particular mono- and di-zygotic twin comparisons allow separation of genetic and environmental influences (although there are certainly some caveats in this approach). Twin studies have done much to inform researchers of the genetic component of alcoholism. Research by Cloninger, Bohman, and Sigvardsson (1981) examined the Stockholm population of adoptees, where they found a distinct pattern of gene-

environment interactions shown to affect the adopted sons' propensity to engage in alcohol abuse. In a U. S. based population study of male twins, it was estimated through the liability threshold method that 48%-58% of risk for developing alcoholism was due to genetic factors (Prescott & Kendler, 1999). When a sample of same and opposite sex twin pairs was analyzed, there was still a large and comparable genetic component of alcoholism risk for women (55%-66%) and men (51%-56%), and the sources of genetic predisposition to alcoholism or alcohol abuse was similar, albeit not identical, between the male and female samples (Prescott, Aggen, & Kendler, 1999). Other studies have found support for a role of inheritance in conferring risk for alcoholism, but to a lesser extent (35% for men and 24% for women), but additionally proposed that certain patterns of inheritance are more likely to have a genetic component (McGue, Pickens, & Svikis, 1992; Pickens et al., 1991). Indeed, more recent research has identified distinct patterns of inheritance of early onset (Kendler, Schmitt, Aggen, & Prescott, 2008) and late onset (Kendler, Gardner, & Dick, 2011) alcohol and substance abuse. It is clear that there are strong genetic and environmental components to alcoholism and identifying these gene-environment interactions, which increases the importance of identifying at risk populations and developing treatments targeted at the underlying causes of alcohol abuse (For review see Enoch, 2012).

Candidate Gene Studies

As discussed above, qualitative and quantitative analyses support the idea that there exists a genetic basis for alcoholism, and that certain traits (response to ethanol, personality traits, metabolic ability, etc.) are heritable. As such, further genetic analysis has served to identify the specific genes that underlie the genetic basis for alcoholism and alcohol-related

behavior. As discussed previously, candidate gene studies assess whether or not specific genes are involved in a given phenotype, in this case alcoholism and related traits. Many studies have examined dopaminergic system genes, given its role in reward and behavioral responses to alcohol. The DAT Val55Ala and Val382Ala allelic variants have a reduced rate of DA uptake and reduced Vmax, respectively (Lin & Uhl, 2003). These changes in amino acid sequence produce a marked change in DA transmission.

Another line of thought is that DAT may be involved in specific aspects of alcoholic symptomology or in particular subtypes of alcoholism. Ueno et al. (1999) found that a polymorphism on the 3'-untranslated region of the DAT gene was associated with alcoholism. As the 3'-untranslated region (UTR) by definition does not directly code for a gene, this association suggests that non-coding elements that alter expression of DAT may have a greater role in the association of DAT with alcoholism. Additional research of this region showed that this polymorphism was associated with higher rates of withdrawal seizure and delirium in alcoholics compared with ethnically matched controls (Sander, Harms, Podschus, et al., 1997). Again, more recent research with a larger sample found an association of single nucleotide polymorphisms in the 3' UTR of the DAT gene with alcoholism, specifically with higher rates of withdrawal seizures after accounting for the severity of alcohol dependence in the subjects (Le Strat et al., 2008). The dopaminergic system, along with DAT specifically, is thought to be involved in many psychiatric disorders, including ADHD and Tourette's Syndrome, along with alcohol dependence. Analysis of individuals meeting diagnostic criteria for the above disorders, compared with controls, revealed no common coding variant of DAT associated with any one of these disorders. The authors suggested that this supports the role of non-coding genetic variants, including variants in the non-coding region of the DAT gene itself, as the potential link between

DAT and alcoholism, as well as other psychiatric disorders (Vandenbergh et al., 2000).

Another type of genetic analysis of the role of DAT in alcoholism involves repeat alleles, which are sequences of DNA that are repeated a specific number of times in tandem. These alleles are formed through errors in transcription or in splicing of exons. Repeat alleles are used as genetic markers for genomic analysis, although the use of single nucleotide polymorphisms in genetic analysis is now more common. A study of Japanese alcoholics showed that alcoholics with the 7-repeat allele for DAT also had the ALDH2*2 genotype, which has been shown to be protective against alcoholism, more often the control sample (Muramatsu & Higuchi, 1995). This finding is of particular interest because the inactive form of ALDH2 is protective against alcoholism, meaning that alcoholics with this form of ALDH2 became alcoholics despite having one protective gene. These findings could suggest some interaction between the 7-repeat allele for DAT and the inactive form of ALDH2 causing increased risk for alcoholism. Another study of a Japanese population found that the 7-repeat allele was found more frequently while the 9-repeat allele was found less frequently in the alcoholic sample (Dobashi et al., 1997). More recent studies have confirmed a role of a DAT polymorphism in alcoholism, but the findings appear to be population specific (Bhaskar, Thangaraj, Wasnik, Singh, & Raghavendra Rao, 2012; Du, Nie, Li, & Wan, 2011), meaning that certain combinations of genotypes (ALDH2*2 and the 7-repeat DAT allele) can confer risk for one group while those alleles may not be present in a group of different ancestry. Moreover, another study utilizing the transmission disequilibrium test found that transmission of the DAT 10 repeat allele occurred more frequently in offspring positive for alcoholism (Samochowiec et al., 2006). Sander and colleagues (1997) showed an association between the 9 repeat allele and severe withdrawal symptoms. Samochowiec et al. (2006) suggested that these findings could be complimentary since the 10

repeat allele was found to be more common in subjects with alcoholism overall as opposed to the 9 repeat allele occurring more frequently in a subset of alcoholics with severe withdrawal symptoms. Parsian and Zhang (1997) attempted to replicate previous associations of the DAT 7-repeat allele with alcoholism, originally identified in Asian populations, in a sample of Caucasians, but found no association between the polymorphisms of the DAT gene and alcoholism. However, the sample did not contain the same specific seven-repeat allele that was associated with alcoholism in the Muramatsu and Higuchi (1995) study.

As the dopaminergic system is implicated in alcoholism, candidate gene studies have been conducted for other dopaminergic genes. Allelic and sequence variants of the DA D_2 receptor have both been linked to alcoholism. One single nucleotide variant identified in the DA D_2 receptor gene that is associated with alcoholism is a guanine substitution in exon 8 of the dopamine D_2 receptor gene in the 3'-untranslated region (Finckh et al., 1997). In a Japanese population, a D_2 receptor allele producing an amino acid sequence variant (Ser311Cys; S311C) was found more frequently in the alcoholic sample compared with controls (Higuchi, Muramatsu, Murayama, & Hayashida, 1994). However, further research in other populations failed to show a significant association between the S311C variant and alcoholism (Finckh et al., 1996; Goldman, Urbanek, Guenther, Robin, & Long, 1997, 1998). As alcoholism is a polygenic trait, the differences between these results could be due to interactions with proteins involved in the expression of the gene, or even involvement with proteins responsible for other aspects of alcohol action and metabolism. The main allelic variation of the DA D_2 associated with alcoholism is the presence of the A1allele (Blum et al., 1990). Further research confirmed a role for the A1 allele in conferring risk for severe alcoholism phenotypes (Blum et al., 1991; Noble, Blum, Ritchie, Montgomery, & Sheridan, 1991; G. Uhl, Blum, Noble, & Smith, 1993). More

recent research of the role of the DA D_2 gene in alcoholism has suggested that the Taq1A1 genotype may confer reduced DA receptor sensitivity as an intermediate phenotype, which can then further affect an individual's propensity to abuse alcohol (Schellekens et al., 2012). Other studies investigating the DA D_2 receptor failed to identify an association with alcoholism (Blomqvist, Gelernter, & Kranzler, 2000; Gelernter & Kranzler, 1999; Gelernter et al., 1991). Other dopaminergic receptors have also been investigated for their involvement in alcohol related disorders. For example, alleles of the DA D_4 receptor were examined for involvement in alcoholism since these alleles have been shown to be involved in novelty seeking (Ono et al., 1997). However, when a sample of male German controls and alcohol dependent subjects were investigated, the allele in question did not seem to affect alcohol seeking behavior (Sander, Harms, Dufeu, et al., 1997).

In summary, when evaluating the results from candidate gene studies it is important to note when allelic differences are identified by using gene markers, and the functional variant is unknown, compared with identifying the studies that identify the functional variant (amino acid change or nucleotide substitution that affects gene expression). While using gene markers is more expeditious than full sequencing, it is possible that these analyses are confounded by differences in ethnic stratification or population specific gene sequences that may not be present in other samples.

Genome-wide Association Studies

Although candidate gene studies have implicated these genes in alcoholism, genome-wide association studies (GWAS), that do not make *a priori* assumptions about the importance of particular genes in alcoholism and examine the entire genome, have generally failed to

identify the DAT and VMAT2 genes in comparisons of alcoholics and matched controls (Edenberg et al., 2010; C. Johnson, Drgon, Walther, & Uhl, 2011; Treutlein et al., 2009). The difference between candidate gene studies and GWAS studies may reflect a variety of factors, including the complex additive genetic mechanisms that may underlie predisposition to alcoholism, genetic heterogeneity and gene-environment interactions. Thus far, it is clear that alcohol-related disorders are highly polygenic. As suggested from the results of some candidate gene studies, factors aside from explicit coding sequence variants are likely to be involved in with the genetic basis of alcoholism. Additionally, it is highly possible that allelic variation in the DAT and VMAT2 genes may contribute to alcoholism only in some populations, or only under some circumstances. One reason to perform animal genetic studies is that they may help identify these complex factors and may produce greater effects than more subtle allelic variations in humans. Moreover, researchers can use transgenic animal models to manipulate genes of interest, identified through genetic analysis of human populations, to clarify the roles of these genes in specific symptomology of alcohol-related disorders through behavioral animal models further.

The familial component of alcoholism could also involve changes in other mechanisms controlling gene expression. The field of epigenetics serves to investigate heritable non-sequenced based changes in DNA structure that affect gene expression—changes that have long lasting effects on an organism and, in some cases, their progeny. An example of these types of epigenetic mechanisms include methylation, acetylation, and phosphorylation, where the respective functional groups are added in place of hydrogens in the DNA or DNA associated proteins (Murray, 1964; For review see Khan, Jahan, & Davie, 2012; Rice & Allis, 2001)

Additional Genetic and Familial Evidence

The two primary enzymes that metabolize alcohol after ingestion are alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH). The genes that code for these respective enzymes have long been implicated in alcoholism. For instance, polymorphisms of the ADH gene have been shown to be protective against alcoholism (Shen et al., 1997; Stamatoyannopoulos, Chen, & Fukui, 1975). Deficiency of the ALDH enzyme is a protective factor against developing alcoholism, especially in Asian populations, as it has been shown that approximately 40% of healthy volunteers have the deficiency while only 2.3% of alcoholics show this deficiency (Harada, Agarwal, Goedde, & Ishikawa, 1983). A similar, but less strong, relationship between ADH/ALDH expression and alcoholism has been identified in non-Asian populations as well (Liu et al., 2011).

Some physiological factors, that may have a genetic basis, have been consistently related to alcoholism. For example, some individuals show an antagonistic physiological response to an alcohol placebo, such as a decrease heart rate and decreased skin conductance response, which is opposite to the physiological effects observed when given actual alcohol (Newlin, 1985; Newlin & Thomson, 1991). Sons of alcoholics tend to show a reduced baseline P3 wave (Olbrich et al., 2002), which is generally accepted as a wave generated when an individual is attempting to identify a novel stimulus, measured through electroencephalography (Begleiter & Porjesz, 1988). Schuckit (1994) suggested that this difference could be indicative of a cognitive deficit that makes it more difficult for an individual to perceive changes in their environment after consuming alcohol, which also results in decreased physiological and subjective reactions to ethanol. It is interesting to note that this decreased reaction applied equally to the positive and negative effects of ethanol consumption—individuals with a family history of alcohol abuse

showed decreased motor incoordination, decreased hormonal response to ethanol, and reported fewer feelings of intoxication, yet their metabolism of ethanol was comparable to controls. There are also consistent differences in background cortical activity of alcoholics compared to controls without other substance abuse issues, especially in the amount, strength, and speed of alpha activity (Ehlers & Schuckit, 1990; Pollock, Gabrielli, Mednick, & Goodwin, 1988), and the speed of beta activity (Gabrielli et al., 1982). These types of high frequency brain waves are localized to frontal areas of the brain, and are involved in relaxed versus attentive states, respectively (For review see Coull, 1998). Finally, a study of men with multiple relatives with alcoholism showed an increase in heart rate elevation and digital blood volume when an unavoidable shock was administered, and these effects were moderated by alcohol to a greater degree when compared with controls (Finn & Pihl, 1988). These characteristic physiological responses as a whole seem to be indicative of a high tolerance for alcohol that increases an individual's risk for developing alcoholism later in life, and relationships between history of family alcohol abuse and these characteristics have been identified. While there are no specific genes suggested by these findings, there is a strong familial component seen in the results of these studies.

Cloninger et al. (1981) proposed a typology that includes two subtypes of alcoholism based on presentation of different abuse traits and risk factors. Risk factors were based on parental history and severity of alcohol abuse, occupational, and criminal history. Other psychological factors that contribute to alcoholism include enhanced negative response to stress (Brady & Sonne, 1999) and high novelty seeking behavior (Bardo, Donohew, & Harrington, 1996). Bardo et al. (1996) suggested the idea that the dopaminergic reward pathway is activated by exposure to novel stimuli (Ljungberg, Apicella, & Schultz, 1992) in a similar fashion to

activation by drugs of abuse. Continuing this line of thought, if an individual is more responsive to the rewarding effects of encountering new stimuli, they might also engage in additional behaviors that produce the same reward, such as drinking alcohol in excess.

In summary, metabolic, physiological, and psychological factors have all been linked to alcoholism in previous research and have been shown to have genetic determinants. Homo- and heterozygote individuals for the inactive form of ADH and ALDH, the enzymes responsible for the breakdown of ethanol, are less likely to develop alcoholism due to their increased adverse response to ethanol even in low concentrations. Characteristic physiological markers have been observed either directly in alcoholic subjects or in their offspring. Some of these physiological differences may contribute to the development of an alcohol abuse disorder. As alcoholism is likely to involve gene-environment interactions, differences in psychological characteristics such as subjective stress response and certain personality traits seem to be more prevalent in subjects with a history of alcohol abuse—personal or familial. Evaluating all of these characteristic differences might allow researchers to understand some of the more complex interactions that underlie the mechanisms behind alcohol abuse.

Genetic research has long aimed to identify connections between genetic variation and behavior, and more recently the importance of genetic differences that affect levels of gene expression have been appreciated. The genetic component contributing to alcoholism and alcohol abuse has been shown to be highly polygenic. Although some large gene effects have been observed, these tend to be limited to certain populations and do not explain the majority of the genetic contribution to alcoholism. Moreover, some specific metabolic, physiological and psychological factors that contribute to an individual's risk for alcoholism have been shown to have a strong genetic component. This evidence only increases the support for animal research

in these types of disorders. By both manipulating genes implicated in alcoholism through human genetic studies, and developing animal behavioral models to represent specific symptomology of alcohol related disorders, researchers are able to clarify the roles of certain genes in alcoholism. Researchers can also investigate under what circumstances genes have a larger or lesser role in alcohol related behavior, and ultimately use animal models of alcohol related disorders to design treatments that can be used to treat these disorders in humans.

Alcohol Consumption Studies in DAT and VMAT2 KO Mice

DAT KO Mice

The effects of transgenic deletion (knockout; KO) of DAT have been extensively studied for a wide range of psychostimulant drugs, and the consequences of DAT KO on dopaminergic neurotransmission have been extensively characterized. The neurochemical and behavioral effects of DAT KO have been characterized thoroughly (Giros, Jaber, Jones, Wightman, & Caron, 1996). Compensatory changes in the dopaminergic system following DAT KO include increased extracellular DA levels, reduced overall DA tissue levels, reduction of DA release, and increased DA synthesis (For review see Gainetdinov, Jones, Fumagalli, Wightman, & Caron, 1998; Jones, Gainetdinov, Wightman, et al., 1998).

Studies using DAT KO mice have provided some information about the potential roles of DAT KO in drug and alcohol dependence, but have produced contradictory and inconclusive results. Savelieva, Caudle, Findlay, Caron, and Miller (2002) used a two-bottle choice paradigm and presented increasing solutions of ethanol (0%, 3%, 6%, 10%, and 15%) for six days at the first three concentrations and then for 10 days at the highest two concentrations. Using these

methods, this study found that female heterozygous DAT KO mice did not differ in ethanol preference or consumption from their wild type (WT) counterparts, while female homozygous DAT KO mice had reduced ethanol consumption and preference. Whereas Savelieva et al. (2002) found no difference in male DAT KO mice compared with heterozygous or WT mice, Hall, Sora, and Uhl (2003) found that heterozygous and homozygous DAT KO male mice had higher preference and consumption of ethanol, more so at higher ethanol concentrations. Hall et al. (2003) also used a two bottle choice paradigm and presented the ethanol in increasing concentrations (2%, 4%, 8%, 12%, 16%, 24%, and 32%) for two to three days per concentration. Morice, Denis, Giros, and Nosten-Bertrand (2010) found that DAT KO mice show increased behavioral sensitization to the locomotor stimulant effects of ethanol, which might be considered to be consistent with the Hall et al. (2003) study. All of these studies show that altered DAT expression leads to alteration of the consumption or other effects of ethanol, although the results are obviously not entirely consistent. This may indicate that there are additional mediating factors affecting the role of DAT in the effects of ethanol consumption. As suggested in analysis of human genetic data, there are potential interactions between the gene of interest and overall genetic background that seem to affect whether or not the DAT gene is associated with alcoholism. Moreover, the difference between methods of accessing ethanol consumption, both duration of exposure and ethanol concentration, could explain some differences between these studies of ethanol consumption in DAT KO mice.

VMAT2 KO Mice

VMAT2 KO mice have also been thoroughly characterized in previous studies. First, homozygous KO of VMAT2 is lethal in mice, so only heterozygous mice are available for

examination (Fon et al., 1997). VMAT2 KO has been shown to reduce tissue content of monoamines (Fon et al., 1997), extracellular monoamine levels, and monoamine release following depolarization or treatment with amphetamine (Wang et al., 1997). Another change in function associated with VMAT2 KO is increased activity of the 5-HT $_{1A}$ autoreceptor in response to reduced 5-HT activity (Narboux-Neme et al., 2011). VMAT2 KO has also been shown to reduce DAT levels in mice (Yamamoto et al., 2007). Heterozygous VMAT2 KO mice show enhanced locomotor effects to treatment with ethanol (Wang et al., 1997).

VMAT2 KO mice have been used in ethanol consumption studies in an attempt to identify the role of VMAT2 in baseline ethanol consumption in mice. Hall et al. (2003) found that heterozygous male VMAT2 KO mice consumed more ethanol at higher concentrations (greater than 16% v/v) than WT controls, while Savelieva, Caudle, and Miller (2006) found that VMAT2 KO mice consumed less ethanol than WT mice. These conflicting results could be due, at least in part, to differing methods. Both studies used two bottle choice paradigms. However, Savelieva et al. (2006) used lower ethanol concentrations (3-15% v/v), and the studies used different strains of KO mice, which could account for the conflicting results if genetic background interacts with the consequences of the gene knockout. Background strain must also be taken into account. First, it has been previously documented that ethanol consumption can vary between strains (Yoneyama, Crabbe, Ford, Murillo, & Finn, 2008), in particular between the two strains that have generally been used to create knockout mice, C57 and 129 mouse strains. Polymorphisms associated with C57 and 129/Sv strains could magnify or obscure effects the desired gene KO has on ethanol consumption itself. A number of genetic polymorphisms have been found to differ between the 129 and C57 strains, and while most polymorphisms are not functional, even differences in a small number of genes could affect ethanol consumption in

ways not specifically related to the knockout (Simpson et al., 1997). When transgenic mice are developed using a mixed genetic background, the parental alleles contribute differentially to the genetic background of the strain. Due partly to the generally small breeding populations available in laboratories, alleles fixate on one of the parental alleles within a short period in time. This fixation seems to happen at random, and without extensive analyses it is not possible to know what specific alleles the transgenic mouse carries from each strain. As C57 mice are known to have higher ethanol preference and consumption compared with other strains, a possible explanation for discrepancies between the Hall and Savelieva research could be a greater number of C57 alleles carried by the Hall KO strains. Although the studies described above did find different consequences of VMAT2 KO, both studies did find altered ethanol consumption, which does warrant further consideration.

Summary

In summary, both DAT and VMAT2 have been implicated in ethanol preference and consumption using transgenic studies, but the effects have not been entirely consistent. In addition to potential differences in the strains, particularly with regard to genetic background, differences in the type of paradigm used to assess ethanol consumption may also be rather important. Blednov and Harris (2008) researched the effects of knockout of the metabotropic glutamate receptor 5 on ethanol consumption under five different consumption paradigms, but only observed genotypic difference under two of the methods (24 hour 4 bottle choice and 4 hour two bottle choice) where the KO mice drank less than their WT counterparts. This finding is of particular importance, because it shows that the specific methods used to access differences in ethanol consumption can affect whether or not difference are observed without any differences in

strain, laboratory, age, or other variables discussed in reference to (Hall et al., 2003), Savelieva et al. (2002), and Savelieva et al. (2006).

Animal Behavioral Models of Ethanol Consumption

Previous research with animal models has sought to elucidate the mechanisms underlying alcohol dependence by examining alcohol consumption and reinforcement. The methods used to investigate these processes include a variety of behavioral models that measure consumption, as well as methods that examine Pavlovian and Instrumental responding. The most common behavioral models that have been used to study these effects of ethanol are drug self-administration, the two-bottle choice paradigm, and conditioned place preference. Drug self-administration, based on operant conditioning principles, involves the animal learning to perform an operant in order to receive access to a reinforcing drug (in this case ethanol). After the behavior is initially established, usually on a fixed ratio 1 (FR1) schedule, the schedule of reinforcement, and other conditions of reinforcement such as the amount of reinforcer per successful response, can be varied to determine the animal's sensitivity to the reinforcer. Other parameters that may be highly relevant to addiction (and cessation of drug taking) can also be examined, such as the rate of extinction (U. C. Campbell & Carroll, 2000). Two-bottle choice paradigms involve having two bottles - one with water and one with a specific concentration of ethanol – freely available to the animal for a specified amount of time (access can be continuous or intermittent). Intermittent access generally leads to voluntary ethanol consumption increase. Usually two-bottle choice testing is done in the home cage, and involves housing the animals singly, which may itself increase ethanol consumption (Hall, Huang, et al., 1998). In two-bottle choice tests both the amount consumed, and the preference for ethanol, are typically measured

(Tordoff & Bachmanov, 2002). Two bottle choice paradigms are often conducted in one of two ways—either *ad lib* over a 24 hour period or for a few hour period at the same time each day or, sometimes, longer intervals. While the 24 hour method is often used to measure consumption, research has shown that the limited access paradigm may better model aspects of human consumption behavior which tends to occur in binges (Le, Ko, Chow, & Quan, 1994). It must be noted though that even in 24 hour access paradigms rodents drink ethanol in short bouts as opposed to continuously (Boyle, Smith, Spivak, & Amit, 1994). Furthermore, often ethanol consumption can be increased by using a limited access paradigm, particularly when exposure is intermittent, leading to a gradual escalation of consumption (Files, Lewis, & Samson, 1994). In this study, rats were presented with ethanol 30 minutes per day for 1-16 sessions each day. Files et al. (1994) noted that limiting this 30 minute access session to once daily produced significant increases in ethanol consumption. Other researchers have identified consumption models that cause mice to drink to intoxicating levels within a 2-4 hour period, when ethanol access is limited to that time (Rhodes, Best, Belknap, Finn, & Crabbe, 2005). These models are important for modeling excessive ethanol consumption. One final commonly used method in alcohol research is conditioned place preference (CPP), a Pavlovian conditioning paradigm that entails pairing a reinforcer—such as alcohol—with one specific environment, while pairing a vehicle injection with a separate specific environment. Subsequently, the reinforcing properties of the drug are assessed by allowing the animal free access to both environments and measuring the preference for the drug-paired environment (Carboni & Vacca, 2003).

Hypothesis

Because of the importance of DAT and VMAT2 in regulating DA and 5-HT function,

and the proposed importance of both these genes and those neurotransmitter systems in alcoholism, the present studies were undertaken to examine the effects of transgenic deletion of these genes on ethanol consumption. Furthermore, because humans do not have complete deletion of these genes, but do show 50% variation in the expression of these genes, wild-type littermate mice expressing normal levels of these genes were compared to heterozygous KO mice that express 50% of wild-type levels, in order to facilitate comparisons to normal human variation in the expression of these genes.

As discussed above, previous research with DAT KO and VMAT2 KO mice has been conducted, but there was noticeable variation with the methods used to evaluate consumption differences, and the results were rather inconsistent. Research in other transgenic models has shown that methods used to access ethanol consumption can affect whether or not differences are observed. With these ideas in mind, the motivation for the present studies was to attempt to access the conditions under which deletion of DAT or VMAT2 as affect ethanol consumption, firstly following a method similar to that of the (Hall et al., 2003) study, and secondly a method that produces escalation of ethanol consumption. Additionally, because genetic background may be highly relevant to the consequences of these gene deletions, all of these studies were conducted in two lines of each knockout strain, the original mixed background strain and a congenic strain.

METHODS

Subjects

The DAT and VMAT2 KO mice used in this study have been described previously (Sora et al., 1998; Takahashi et al., 1997), and have been maintained on a mixed C57BL/6J-129SVev

genetic background. In addition, these studies also examine congenic C57BL/6J DAT KO and congenic C57BL/6J VMAT2 KO strains, termed DAT BX and VMAT2 BX, respectively, produced by more than 10 generations of backcrosses of C57BL/6J mice to the two original strains. Briefly, gene KO using transgenic techniques is produced by replacing a portion of the gene of interest with an alternative sequence (not found in mice, usually a bacterial gene sequence, termed the knockout "construct") in mouse embryonic stem (ES) cells. In order to target a specific gene, a viral vector is made that contains the bacterial sequence flanked by a sequence containing a homologous region to a portion of the DNA to be altered. In some of the ES cells, this homologous region produces homologous recombination, and consequently the replacement of the target gene sequence with the knockout construct. The ES cells are then selected for ones in which this recombination has occurred, and those cells are injected into mouse blastocysts, producing chimeric offspring when implanted into pseudo-pregnant female mice. The chimeric offspring are then bred to WT females and the offspring examined for transmission of the gene (which occurs if cells containing the transgene produce gonadal tissue in the chimeric mice).

The experiments described here used wild type (WT) and heterozygote (hKO) mice of both sexes from the 4 strains described above. Thus, the design of these experiments involves comparison of mice with heterozygous KO of the DAT and VMAT2 genes on two genetic backgrounds (mixed background, DAT and VMAT2, and congenic backgrounds, DAT BX and VMAT2 BX), resulting in 4 comparisons for each behavioral experiment: DAT WT vs. DAT hKO, DAT BX WT vs. DAT BX hKO, VMAT2 WT vs. VMAT2 hKO, and VMAT2 BX WT vs. VMAT2 BX hKO. Results from males was compared with results of females for all strains. The total number of mice studied was N=10 per experimental condition for consumption experiments

and N= 9-14 per experimental condition for escalation experiments. All transgenic mice were bred in the Triad Breeding Facility at the National Institute on Drug Abuse (NIDA), and all behavioral testing occurred in NIDA facilities. All experiments were conducted in accordance with AALAC guidelines under protocols approved by the NIDA Animal Care and Use Committee.

Breeding, genotyping and housing procedures are similar to those initially described in Hall et al. (2003). Litters were weaned at 21 days of age, and segregated by sex. At weaning, 0.2 cm tissue samples (from a small ear punch prior to insertion of ear tags for identification) were taken for genotyping by PCR and gel electrophoresis. Tissue samples were incubated for 3 hours at 55°C in tail buffer (50 mM Tris, pH 8.0; 100 mM EDTA; 100 mM NaCl; 1% SDS) containing Protease K (10 mg/ml). Supernatants were removed and lysis buffer added (0.32 M sucrose; 10 mM Tris, pH 7.5; 5 mM MgCl; 1% Triton X-100). After centrifugation the supernatant was removed and the tail DNA solution was used for PCR using E2tak buffer (Clontech Laboratories), 1 mM dNTP mix (Clontech Laboratories), BSA (New England Bio Labs, 10 mg/mL), Dimethyl Sulfoxide (Sigma Chemical Co.) and Takara E2tak DNA Polymerase (Clontech Laboratories).

Oligonucleotides (10 μM) for VMAT2 genotyping included a forward primer located outside the knockout region (5’ GAA TGT GCA AGT TGG GCT GCT G 3’), a reverse primer for the VMAT2 gene located in the region of the WT gene (not present in the KO construct; 5’ GTG CCC AGT TTA TGT AGC ATT GG 3’), and a reverse primer for the NEO gene that is present in the knockout construct (5' TCG ACG TTG TCA CTG AAG CGG 3'). Amplimers thus produced from wild-type DNA were 600 bp and amplimers from the KO DNA were 900 bp. Homozygous WT mice thus produced only the WT band, homozygous knockout mice produced

only the KO band and heterozygous mice produced both bands.

For DAT genotyping, oligonucleotides (10 μM) included a forward primer located outside the deleted region (5' AGT GTG TGC AGG GCA TGG TGT A 3'), a reverse primer for the WT DAT gene located in the region (not present in the KO construct; 5' TAG GCA CTG CTG ACG ATG ACT G 3'), and a reverse primer for the NEO gene that is present in the knockout construct (5' CTC GTC GTG ACC CAT GGC GAT 3'). Amplimers thus produced from wild-type DNA were 500 bp and from KO DNA were 600 bp. Again, homozygous WT mice thus produced only the WT band, homozygous knockout mice produced only the KO band and heterozygous mice produced both bands.

Experiment 1: Consumption of ascending concentrations of ethanol

After 1 day of habituation to single housing, male (M) and female (F) mice of both genotypes (WT, hKO) from each mice mouse strain (VMAT, VMATBX, DAT, DATBX; n=10 per experimental condition) were weighed and given free access to ethanol and water continuously for 10 days in two-bottle, 24 hr. access, home-cage preference tests. Ethanol concentrations were changed every 2 days in the following progression: 2%, 4%, 8%, 16%, and 32% (v/v). Both ethanol and water bottles were weighed daily. To control for side preferences, the placement of bottles was switched each day. Consumption was expressed as volume ethanol and water consumed, total fluid consumption, preference, grams ethanol consumed per kilogram body weight per day (g/kg/day) ethanol consumption.

Experiment 2: Escalation of Ethanol Consumption using an Intermittent Access Paradigm

When rodents are presented with ethanol intermittently, they have been shown to

"escalate" (increase) consumption of ethanol. The precise experimental parameters that lead to "escalation" vary with species, strain, sex, etc. Thus, conditions that produced escalation of ethanol consumption in the mouse strains used in the present studies were established in preliminary experiments (data not presented). Subsequently, male and female mice of each genotype (WT, hKO) for each strain (n=12 per experimental condition for the VMAT and VMAT BX strains, and n=9-14 for each experimental group for the DAT and DAT BX strains) were subjected to an "escalation" paradigm in which ethanol was available intermittently, 2 days per week for 3 weeks, at a concentration of 8%. On these days, food and water were also freely available. On the intervening days, food and water were again available *ad libitum*, but not ethanol. On test days, ethanol and water bottles were weighed before and after presentation and consumption expressed as volume ethanol and water consumed, total fluid consumption, preference, g/kg/day ethanol consumption, and percent change in ethanol consumption from Day 1.

Data Analysis

Experiment 1

Data for each strain (VMAT, VMATBX, DAT, and DATBX) were analyzed separately by analysis of variance (ANOVA) with the between-subjects factor of GENOTYPE (WT vs. hKO) and SEX (M vs. F), and the within-subjects factor of CONCENTRATION. In this initial ANOVA sex was included as a factor because ethanol consumption is well known to differ between male and female mice (Blanchard, Steindorf, Wang, and Glick, 1993; Savelieva, Caudle, Findlay, Caron, and Miller, 2002). Following this initial analysis subsequent ANOVA were performed separately on data from male and female mice, to further determine the nature of

the effects. Fisher's PLSD was used for *post hoc* comparisons between individual means.

Experiment 2

Data for each strain - VMAT, VMATBX, DAT, and DATBX - were submitted to ANOVA with the between-subjects factors of GENOTYPE (WT vs. hKO) and SEX (M vs. F) and the within subjects factor of SESSION. Initial ANOVA included sex as a factor due to previous research showing differences in consumption between male and female mice (Blanchard, Steindorf, Wang, & Glick, 1993; Savelieva et al., 2002). Once a significant effect of SESSION was identified in initial ANOVA, *post hoc* 1 way ANOVA were performed for each experimental group to determine which groups demonstrated significant escalation. Fisher's PLSD was used for *post hoc* comparisons between individual means when ANOVA demonstrated significance of the factors. To further illustrate the escalation phenomenon the percent increase over the course of the study was calculated as a relative change versus the first day (this also serves to take into account potential initial differences in consumption).

Hypothesis

Experiment 1

Based on previous experiments, and the analysis presented above, it was hypothesized that both DAT KO and VMAT2 KO mice would have increased ethanol consumption, in an ethanol concentration and sex dependent manner. The elevated consumption of ethanol characteristic of the C57BL/6J strain (McClearn & Rodgers, 1959; Belknap, Crabbe, & Young, 1993), the background strain of the congenic strains, was hypothesized to increase the magnitude of these effects.

Experiment 2

Once conditions were defined under which escalation of ethanol consumption was observed, it was hypothesized that the KO mice would escalate at a greater rate compared to their WT counterparts. Females were expected to consume more than males. Again, it was expected that these effects would be magnified in the congenic strains due to the characteristically greater consumption of the C57BL/6J strain.

RESULTS

Experiment 1A: DAT KO

There was no significant effect of GENOTYPE, nor any interactions of GENOTYPE for any measures analyzed ($p>0.05$). As was expected, the concentration of ethanol had a large effect on the amount of ethanol consumed. Thus, ANOVA for the volume of ethanol solution, water, and total fluid consumed revealed significant effects of CONCENTRATION ($F [4,36] = 19.1$, $p<0.0001$; $F [4,36] = 42.0$, $p<0.0001$; $F [4,36] = 25.3$, $p<0.0001$; Figs. 1A-1C).

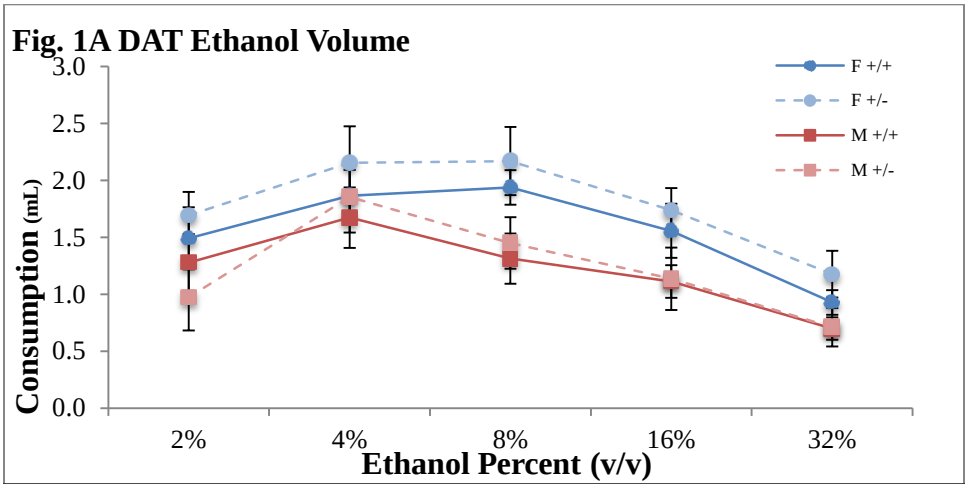


Figure 1A. Consumption of different volumes of ethanol solutions (2% to 32% v/v) in male (M) and female (F) DAT WT and DAT hKO mice. There was a significant effect of concentration on ethanol consumption for all groups ($F [4,36] = 19.1$, $p<0.0001$), and an effect of SEX ($F [1,36] = 6.9$, $p<0.02$) where females drank more ethanol solution than males.

For ethanol solution consumption, an effect of SEX was observed (F [1,36] = 6.9, p<0.02; Fig 1A), where females consumed more ethanol solution than males, particularly at moderate ethanol concentrations. When water consumption was analyzed an effect of SEX (F [1,36] = 9.7], p<0.004) was also observed, however, consistent with reduced consumption of ethanol solutions in males, the opposite was observed for water where males consumed more than females.

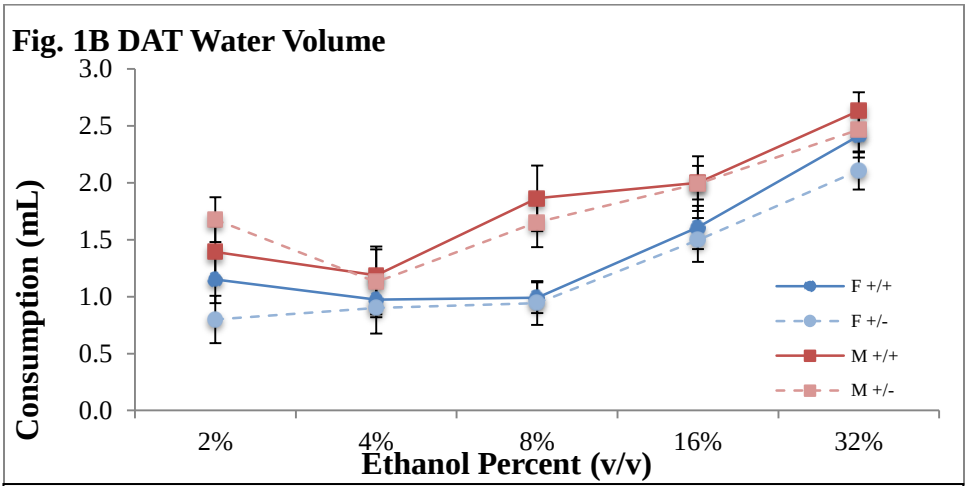


Figure 1B. Water consumption (mL) in male (M) and female (F) DAT WT and DAT hKO mice. Water consumption was inversely related to ethanol solution consumption, and was significantly affected by CONCENTRATION (F [4,36] = 19.5, p<0.0001) and SEX (F [1,36] = 6.9, p<0.02), where M drank more than F.

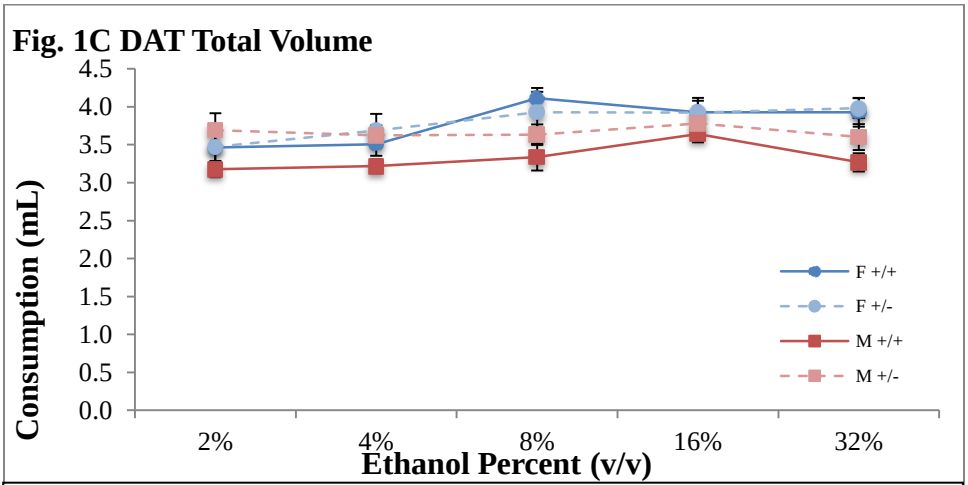


Figure 1C. Total fluid consumption increased with concentration. ANOVA showed a significant effect of CONCENTRATION (F [4,36] = 25.3, p<0.0001) on total fluid

Overall, mice consumed more ethanol solution as the concentration was initially increased, and then less at higher concentrations (overall ethanol consumption, of course did not decrease, as reflected in the g/kg/day measure of ethanol consumption, below). Generally, water consumption varied in a reciprocal manner to consumption of ethanol solutions, although more so at higher ethanol concentrations resulting in slightly increased total consumption. To some extent, this may reflect dehydration produced by these high ethanol concentrations, or intentional dilution of ethanol by concurrent water consumption to effectively achieve a more optimal or preferred concentration. Given the above description, it is not surprising that ethanol preference was also affected by CONCENTRATION ($F [4,36] = 25.4$, $p<0.0001$; Fig. 1D), increasing slightly as the concentration increased and then being reduced at higher ethanol concentrations. On average, females showed about 60% preference and males showed 40% preference for 2% ethanol solution, males showed 60% and females showed 65% preference for 4% ethanol solution, females maintained 65% preference and males showed 45% preference for 8% ethanol solution. Ethanol preference began to reduce after the 8% solution—females showed 50% preference and males showed 35% preference for 16% ethanol solution, and finally females showed about 35% preference and males showed 20% preference for 32% ethanol solution. It should be noted that there was a non-significant trend for female hKO mice to have increased preference compared with female WT mice. It follows that a significant effect of SEX ($F [1, 36] = 9.9$, $p<0.004$) was observed, reflective of increased ethanol preference in female mice, particularly at higher ethanol concentrations, consistent with previous findings (Hall et al., 2003; Savelieva et al., 2006).

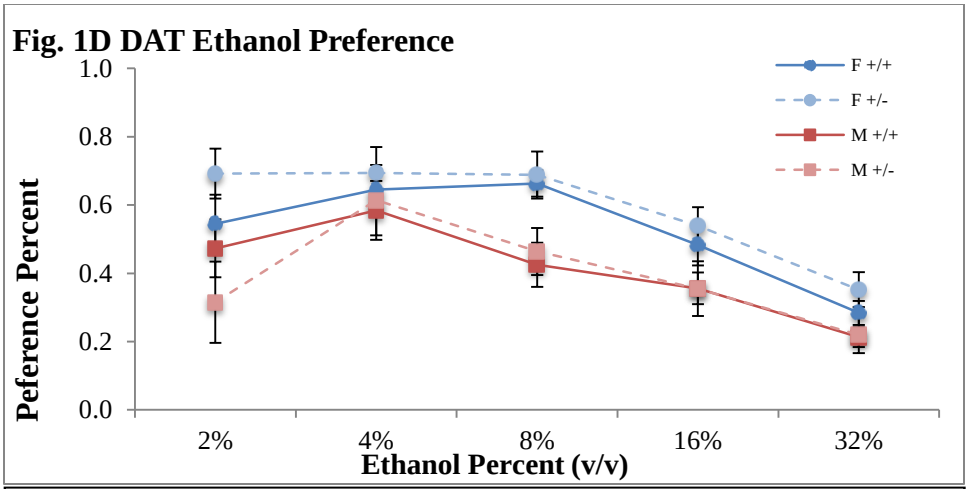


Figure 1D. Females had higher preference than males, and there was a significant effect of SEX on ethanol preference (F [1,36] = 9.8, p<0.004). Overall there was also an effect of CONCENTRATION (F [4,36] = 25.4, p<0.0001).

Ethanol consumption as g/kg/day, thereby taking into account differences in weight, female mice were found to consume much more ethanol than males, particularly at higher concentrations. There was a significant effect of SEX (F [1, 36] = 34.6, p<0.0001; Fig. 1E), CONCENTRATION (F [4, 36] = 65.9, p<0.0001; Fig 1E), and a significant CONCENTRATION x SEX interaction (F [4, 36] = 8.3, p<0.0001; Fig 1E). However, in this case there was neither a significant effect of GENOTYPE (F [1, 36] = 1.03, p<0.317), nor any significant interactions of the other factors with GENOTYPE.

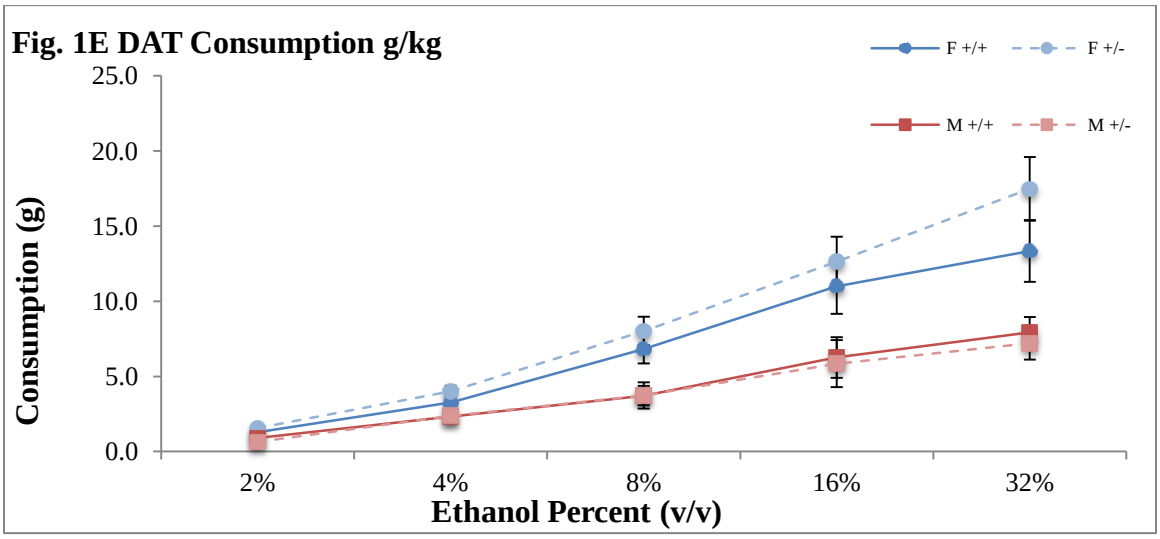


Figure 1E. Females consumed more ethanol than males, particularly at higher concentrations. This is represented in the observed effects of SEX (F [1, 36] = 34.6, p<0.0001), CONCENTRATION (F [4, 36] = 65.9, p<0.0001), and CONCENTRATION x SEX (F [4, 36] = 8.3, p<0.0001).

Due to the differences between sexes observed in some of the measurements in the initial analyses above, separate analyses were also performed for each sex. When ethanol solution consumption was analyzed in female mice, a significant effect of CONCENTRATION was observed ($F [4,18] = 9.7$, $p<0.0001$). *Post hoc* comparison of means, independent of genotype, revealed significant increase in ethanol solution consumption between 2% and 4%, and 2% and 8% concentrations ($p<0.03$), and significant decrease in ethanol solution consumption between 8% and 16% and 8% and 32% ($p<0.03$ and $p<0.0001$ respectively). There was a significant effect of CONCENTRATION on water consumption ($F [4,16] = 31.8$, $p<0.0001$) as well, and *post hoc* comparisons of means, independent of genotype, showed that there were significant increases in water consumption for 16% ($p<0.0002$) and 32% ($p<0.0001$) ethanol when compared with the lowest concentration. Total fluid consumption increased across the experiment, and indeed there was a significant effect of CONCENTRATION ($F [4,18] = 16.2$, $p<0.0001$), and *post hoc* analyses revealed significant increases between the first concentration and second ($p<0.0003$), and all subsequent concentrations ($p<0.0001$). Given the above description, it follows that ethanol preference stayed about the same for the first three ethanol concentrations (2%, 4%, and 8% v/v), and then decreased significantly for 16% ($p<0.04$) and 32% ($p<0.0001$) ethanol solutions. Finally, ethanol consumption calculated as g/kg/day increased steadily as the ethanol solution concentration increased, not quite reaching an asymptote at the highest concentration, particularly in female mice. *Post hoc* analysis showed that this increase was significant for the change between 2% and 8%-32% ($p<0.0001$) solutions. There was a trend for female DAT hKO mice to consume more ethanol than female WT mice, particularly at the highest ethanol concentration, that did not reach statistical significance ($p>0.05$).

Similar analyses were performed for the data from male DAT mice. Analysis of ethanol solution consumption data showed a significant effect of CONCENTRATION ($F [4,18] = 10.6$, $p<0.0001$). *Post hoc* analyses showed a significant increase in ethanol solution consumption from the 2% solution for the 4% ($p<0.03$) and 8% ($p<0.01$) solutions, and a significant decrease in consumption of the 32% ($p<0.005$) solution compared with the 2% solution. Water consumption was inversely related to ethanol solution consumption, ANOVA showed a significant effect of CONCENTRATION ($F [4,18] = 16.4$, $p<0.0001$), and *post hoc* analysis revealed there was a significant increase of water consumption with the 16% ($p<0.0002$) and 32% ($p<0.0001$) solutions compared with the 2% ethanol solution. Total fluid consumption steadily increased for males, with ANOVA showing a significant effect of CONCENTRATION ($F [4,18] = 10.0$, $p<0.0001$) and *post hoc* analyses showing increases from the initial 2% concentration for the 4% ($p<0.0003$) solution, and all subsequent solutions ($p<0.0001$). Similar to the other measures, preference was significantly affected by CONCENTRATION ($F [4,18] = 17.4$, $p<0.0001$). When preference was described in the initial ANOVA, a non-significant initial difference in preference was noted for the 2% solution between WT and hKO males. *Post hoc* comparison of means, independent of genotype, showed a significant increase in preference from 2% to 8% ethanol solution ($p<0.04$), as maximum preference was observed for 4% solution (60% average preference for both WT and hKO mice), subsequent decreases were compared with the 4% solution. There were significant decreases in preference from 4% to 16% ($p<0.003$) and 32% ($p<0.0001$). Finally, consumption calculated as g/kg/day was significantly affected by CONCENTRATION ($F [4,18] = 27.3$, $p<0.0001$). *Post hoc* comparison of means, independent of genotype, showed that consumption significantly increased. Increase from 2% to 4% was significant ($p<0.04$), as were increases to 8% ($p<0.002$), 16% and 32%

($p<0.0001$).

Experiment 1B: DAT BX

Overall, DAT BX mice had higher levels of consumption of ethanol solutions and higher initial levels of total fluid consumption compared with DAT mice. Specifically, higher ethanol preference was noted for 8% and 16% ethanol solutions. No effect of SEX was observed for DAT BX mice like the results observed in the DAT baseline consumption experiment. These results are consistent with our hypothesis that congenic strains would show elevated levels of consumption and preference compared to mixed strains. Male DAT BX mice showed marked increases in ethanol consumption for 8%-32% consumption as g/kg/day, but females maintained approximately similar levels of consumption for 2%-16% ethanol solutions, but DAT BX females reduced their g/kg/day consumption for 32% ethanol solution compared with DAT females. See analyses for each measure for more specific comparisons.

ANOVA for the volume of ethanol solution consumption showed significant effects of CONCENTRATION ($F [4,36] = 15.5$, $p<0.0001$ and CONCENTRATION x SEX ($F[4, 36] = 2.5$, $p<0.042$; Fig 2A), the interaction apparently due to slightly elevated female consumption at the middle concentration, and slightly elevated male hKO consumption at 2% and 32% ethanol solutions. All groups showed increasing consumption from 2% to 8% ethanol, for which the greatest volume of ethanol solution was consumed. As the concentration increased above 8%, there was a decrease in the volume to ethanol solution consumed for all groups.

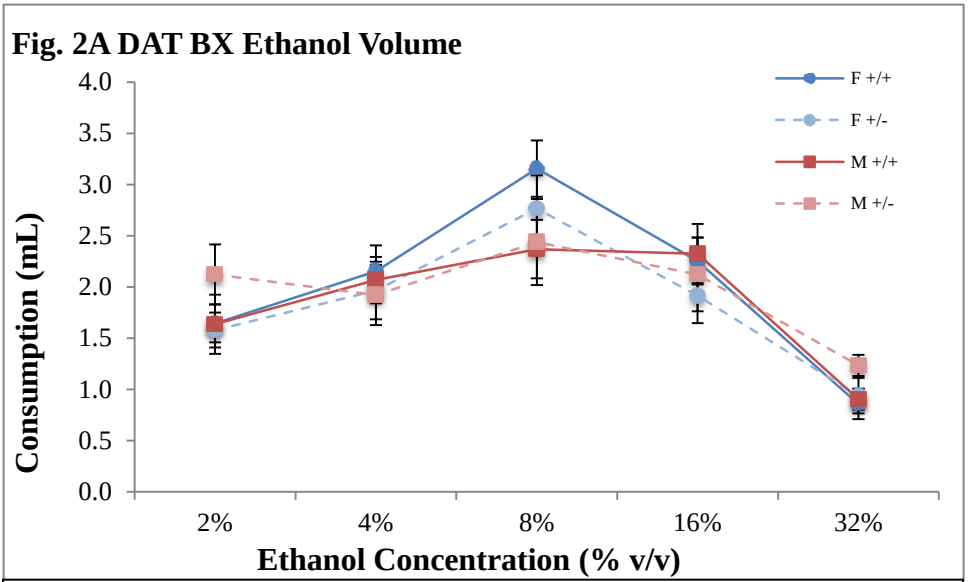


Figure 2A. Ethanol solution consumption followed a somewhat inverted U shaped curve. Females consumed more moderate ethanol solution (8% v/v) than males, and significant effects of CONCENTRATION ($F [4,36] = 15.5$, $p<0.0001$ and CONCENTRATION x SEX ($F[4, 36] = 2.5$, $p<0.042$) were observed.

Water consumption again showed an opposite pattern to that for the volume of ethanol solution consumed, showing decreased water consumption when there was increased consumption of ethanol solutions, and increased water consumption at lower levels of ethanol solution consumption, resulting in a somewhat U shaped curve. The above description is corroborated by the ANOVA of water volume consumption, which showed a significant effect of CONCENTRATION ($F [4,36] = 14.6$, $p<0.0001$; Fig 2B).

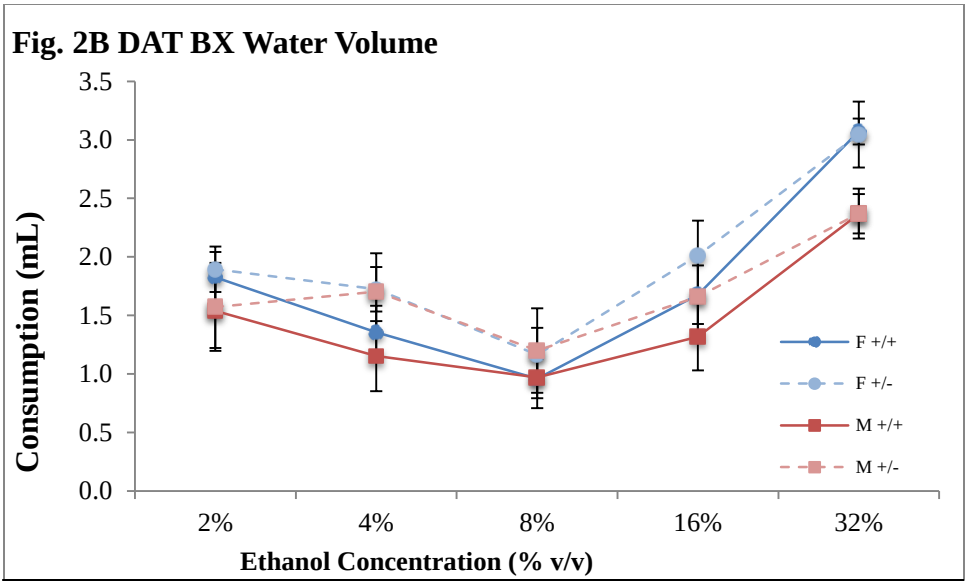


Figure 2B. Water consumption of DAT BX mice was not different between groups, but analysis showed a significant effect of CONCENTRATION (F [4,36] = 14.6, p<0.0001), as water consumption was inversely related to ethanol solution consumption.

Total volume of fluid consumption was affected by SEX (F [1,36] = 4.4, p<0.04; Fig. 2C), CONCENTRATION (F [4, 36] = 13.4, p<0.0001), and CONCENTRATION x SEX (F [4, 36] = 6.6, p<0.0001). Females showed increasing total fluid consumption throughout the experiment, while males seemed to show a more consistent total fluid consumption across ethanol concentrations.

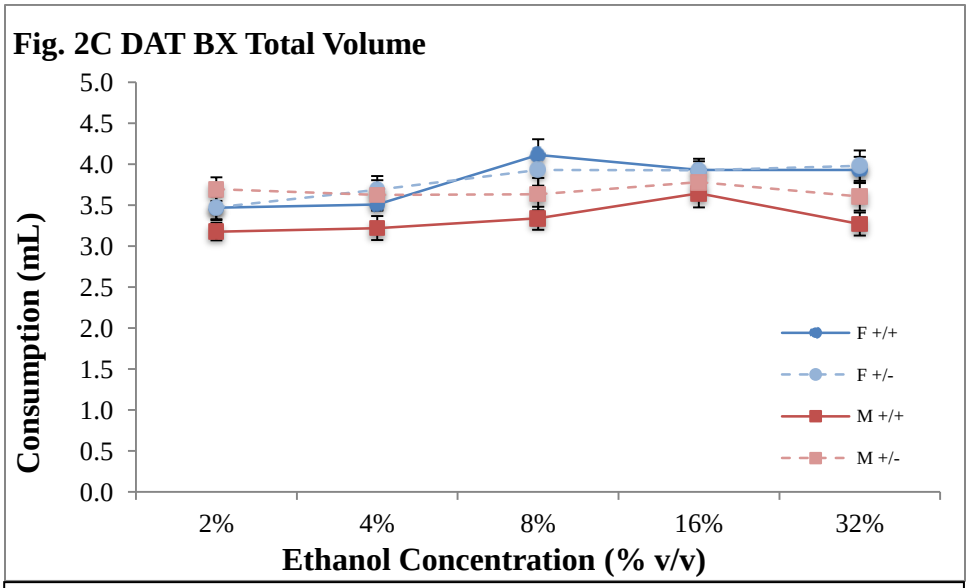


Figure 2C. Total volume of fluid consumption was affected by SEX (F [1,36] = 4.4, p<0.04), CONCENTRATION (F [4, 36] = 13.4, p<0.0001; Fig. 2C), and CONCENTRATION x SEX (F [4, 36] = 6.6, p<0.0001)

The ethanol preference was significantly affected by CONCENTRATION ($F [4,36] = 39.22$, $p<0.0001$; Fig 2D), which proceeds logically from the results for the volume of ethanol solution and water consumed, where there was peak preference for all groups at 8% ethanol, with increasing preference below 8% and decreasing preference above 8%. DAT BX mice showed increased preference for the 8% and 16% (on average) solutions compared with the DAT mice—DAT BX mice showed 70% preference for 8% ethanol solution while DAT females showed 65% preference and males showed 45% for this solution. At 16% ethanol solution, DAT BX mice showed 60% preference compared to 50% preference for DAT females and 35% preference for DAT males.

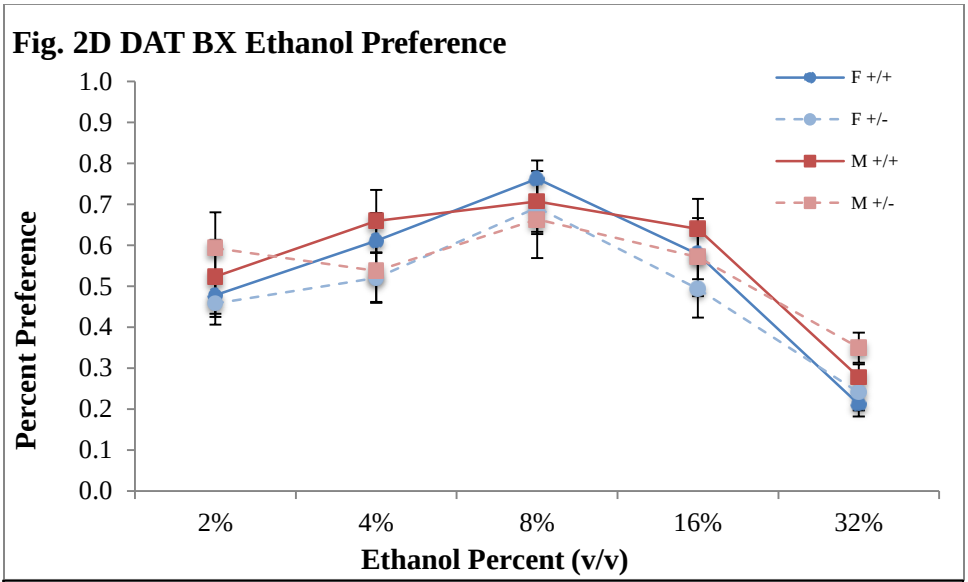


Figure 2D. Ethanol preference was similar across groups and dependent on ethanol concentration, as shown by a significant effect of CONCENTRATION ($F [4,36] = 39.22$, $p<0.0001$).

In the case of ethanol consumption calculated as g/kg/day, females started at approximately the same level of consumption as males at low concentrations, but increased consumption more than males as the ethanol concentration was increased. This increase is most

obvious at the 8% ethanol concentration. Males increased their consumption to match the higher female consumption at the higher concentrations. Ethanol consumption expressed as g/kg/day was significantly affected by SEX (F [1, 36] = 9.8, p<0.004; Fig 2E) and CONCENTRATION (F [4, 36] = 93.6, p<0.0001; Fig 2E), as well as a significant CONCENTRATION x SEX interaction (F [4, 36] = 28.6, p<0.01; Fig 2E).

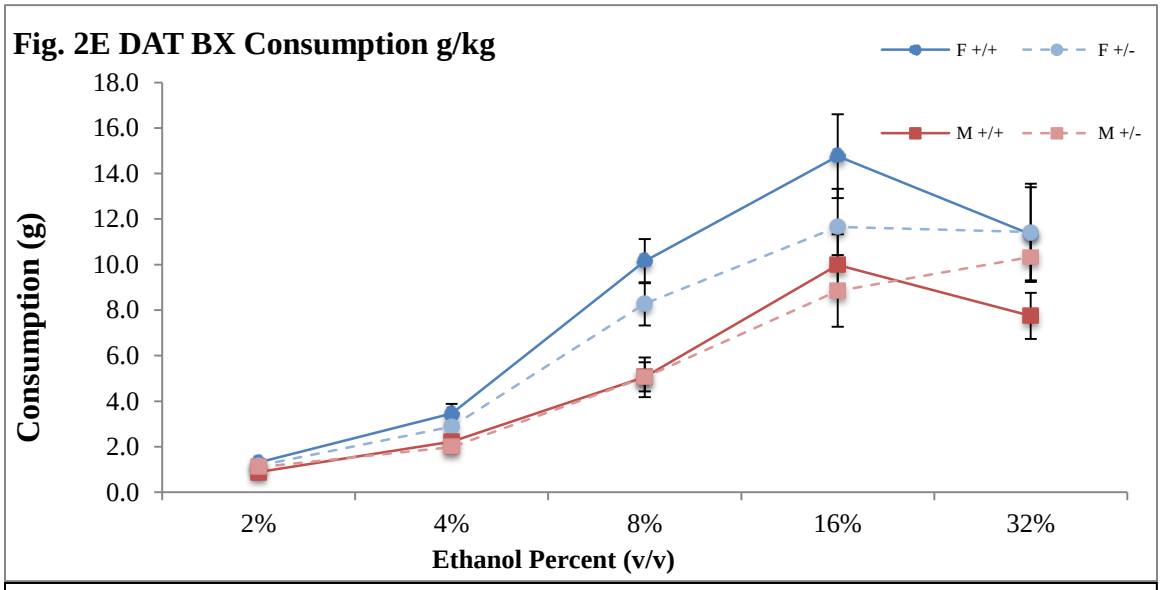


Figure 2E. Ethanol consumption differed between males and females at higher concentrations and there were significant effects of SEX (F [1, 36] = 9.8, p<0.004) and CONCENTRATION (F [4, 36] = 93.6, p<0.0001), as well as a significant CONCENTRATION x SEX interaction (F [4, 36] = 28.6, p<0.01).

Post hoc ANOVAs were performed separately for each sex after significant effects of SEX were identified in the initial ANOVA. As described for the DAT KO strain, female mice increased ethanol solution consumption from 2% to 8% concentrations, and then decreased ethanol solution consumption for 16% and 32% solutions, and there was a significant effect of CONCENTRATION (F [4,18] = 30.0, p<0.0001) on ethanol solution consumption. *Post hoc* analyses showed significant increases for 2% to 4% (p<0.03) and 2% to 8% (p<0.0001), and significant decreases from 8% to 16% and 32% (p<0.0001). Water consumption showed similar patterns to DAT mice, where water consumption decreased as ethanol solution consumption

increased, and water consumption increased as ethanol solution consumption decreased. There was a significant effect of CONCENTRATION ($F [4,18] = 28.1$, $p<0.0001$) on water consumption. *Post hoc* analysis showed significant decreases from 2% to 8% solutions ($p<0.0001$), and significant increases in water consumption from 8% to 16% ($p<0.0003$) and 32% ($p<0.0001$) when means were compared independent of genotype. There were no significant effects or interactions involving SEX or GENOTYPE for total fluid consumption or ethanol preference in the initial ANOVA, so *post hoc* analysis was not warranted. Finally, ethanol consumption calculated as g/kg/day steadily increased across concentrations, and there was a significant effect of CONCENTRATION ($F [4,18] = 45.3$, $p<0.0001$). *Post hoc* analysis showed significant increases between 2% and 8% and all subsequent solutions ($p<0.0001$). There was no effect of GENOTYPE ($F [1,18] = 0.829$, NS) on this or any other measure.

As with the previous results discussed for female DAT BX mice, *post hoc* analyses were not presented for males as no significant effects or interactions involving SEX or GENOTYPE were observed in the initial ANOVA. Therefore, *post hoc* analyses of total fluid consumption and ethanol preference are not presented. For subsequent measures, males showed similar patterns to females. Ethanol solution consumption increased from 2% to 16% concentrations, and decreased for 32% concentration, with an effect of CONCENTRATION ($F [4,18] = 10.9$, $p<0.0001$) on ethanol solution consumption for males. *Post hoc* mean comparisons, independent of genotype, showed significant increases from 2% solution to 4% solution ($p<0.025$), 8% solution ($p<0.0001$), and 16% solution ($p<0.02$), and significant decrease from 8% solution to 16%-32% solutions ($p<0.0001$). Water consumption was inversely related to ethanol solution consumption, and there was an effect of CONCENTRATION ($F [4,18] = 12.8$, $p<0.0001$) on water consumption. Significant reduction in water consumption was observed between 2% and

8% solutions ($p<0.0001$), and significant increases in water consumption were observed between 8% solution and 16%-32% solution ($p<0.0002$) when means were compared, independent of genotype. There was a trend towards increased total fluid consumption in heterozygous KO males, but the effect of GENOTYPE ($F [1,18] = 3.2$, $p<0.089$) was not significant. There was a significant effect of CONCENTRATION ($F [4,18] = 3.6$, $p<0.01$) on total fluid consumption, and subsequent analysis showed significant increases in total fluid consumption from 2% solution to 8%-32% solutions ($p<0.0001$) after mean comparison, independent of genotype. As before, ethanol preference was similar to ethanol solution consumption. There was an effect of CONCENTRATION ($F [4,18] = 14.2$, $p<0.0001$) on ethanol preference. There was significant increase of ethanol preference from 2% solution to 8% solution ($p<0.0001$) and there was significant decrease in preference from solution 8% to 16% ($p<0.0004$) and 32% ($p<0.0001$) solutions, as determined by *post hoc* means comparisons, independent of genotype. Finally, ethanol consumption in g/kg/day increased consistently across all concentrations. There was a significant effect of CONCENTRATION ($F [4,18] = 55.1$, $p<0.0001$) on this measure, and *post hoc* comparison of means showed this that increase was significant between the 2% solution and 8%-32% ($p<0.0001$) solutions. There was no significant effect of GENOTYPE ($F [1,18] = 0.111$, NS) on this measure, or any other measures for male DAT BX mice.

Experiment 1C: VMAT KO

In contrast to the original hypotheses, there was no significant effect of genotype or sex on any of the behavioral measures in this experiment. As can be seen in Figs. 3A to 3E, all groups show a very similar pattern of behavior for all measures. Overall levels of ethanol solution consumption were similar to levels observed in the mixed DAT strain, meaning that

higher levels of ethanol solution consumption were observed in DAT BX mice, consistent with the hypothesis for elevated consumption and preference in congenic strains. This strain showed a similar pattern of slightly increasing total fluid consumption, as seen in the DAT strain, although this strain started at about 3 mL/day compared to 2.5 mL/day in the DAT strain. DAT mice showed a similar inverted U shaped curve in terms of preference. There were no sex-dependent effects as seen with VMAT mice. The levels of preference were most consistent with female DAT preference. Steadily increasing consumption as g/kg/day was observed for all groups of DAT mice in a similar pattern to VMAT mice, although overall levels of DAT consumption were lower than VMAT mice. Specific differences will be discussed along with analyses of each measure.

All groups showed increased consumption of ethanol solutions at low concentrations, with maximum consumption occurring at 8% ethanol, and decreasing consumption at the higher concentrations of ethanol producing a somewhat inverted U-shaped curve. ANOVA confirmed this description revealing a significant effect of CONCENTRATION ($F [4,36] = 16.7$, $p<0.0001$; Fig 3A). Levels of ethanol solution consumption were similar to levels observed with female DAT mice for all ethanol solutions.

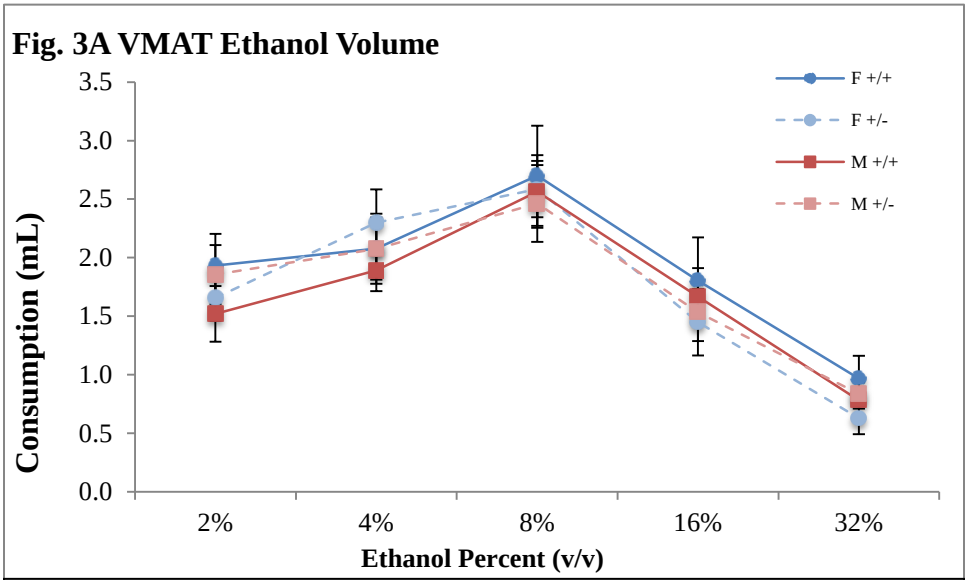


Figure 3A. All groups showed similar patterns of fluid consumption to other strains, and VMAT mice consumed similar amounts of ethanol solution as DAT mice. A significant effect of CONCENTRATION ($F [4,36] = 16.7$, $p<0.0001$) was observed.

As observed for other strains, water consumption decreased when consumption of ethanol solutions increased, and water consumption increased when consumption of ethanol solutions decreased, which resulted in a somewhat U-shaped curve for water consumption. Analysis of water consumption showed a significant effect of CONCENTRATION (F [4, 36] = 55.6, p<0.0001; Fig 3B).

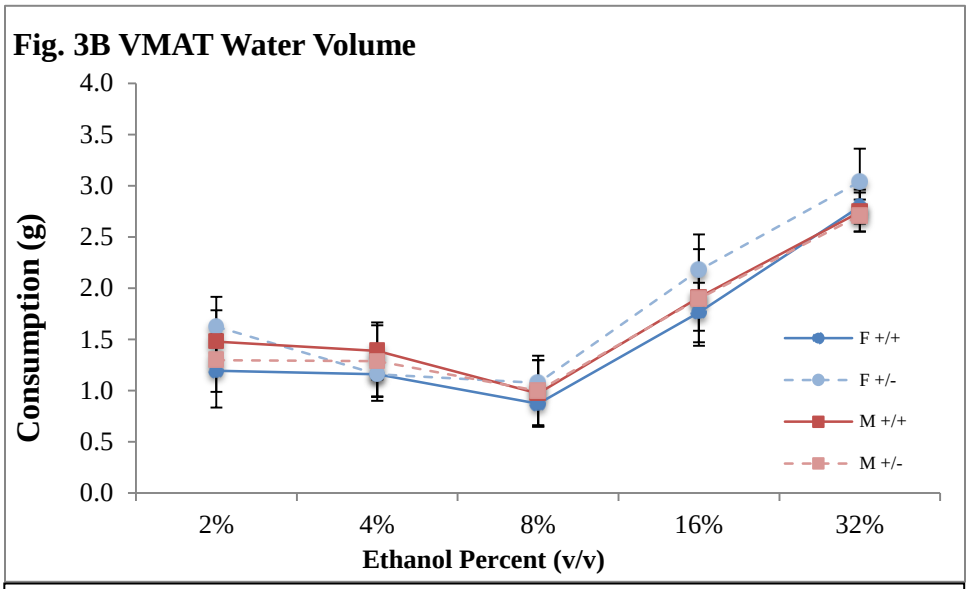


Figure 3B. Water consumption was inversely related to ethanol solution consumption, so that water consumption decreased up to the 8% condition and increased thereafter. Analysis showed a significant effect of CONCENTRATION (F [4, 36] = 55.6, p<0.0001)

All groups increased their total fluid consumption slightly for the duration of the experiment, as the concentration of ethanol solution was increased. As previously stated, there were changes in both the volumes of ethanol and water consumed as the ethanol concentration was increased, but no differences between experimental groups. As suggested previously, the increase in total volume consumed could be an effect of the mice drinking more water for compensatory reasons as the concentration of ethanol was increased. The effect of CONCENTRATION on total volume of fluid consumption was confirmed by ANOVA (F [4,36] = 12.2, p<0.0001; Fig 3C).

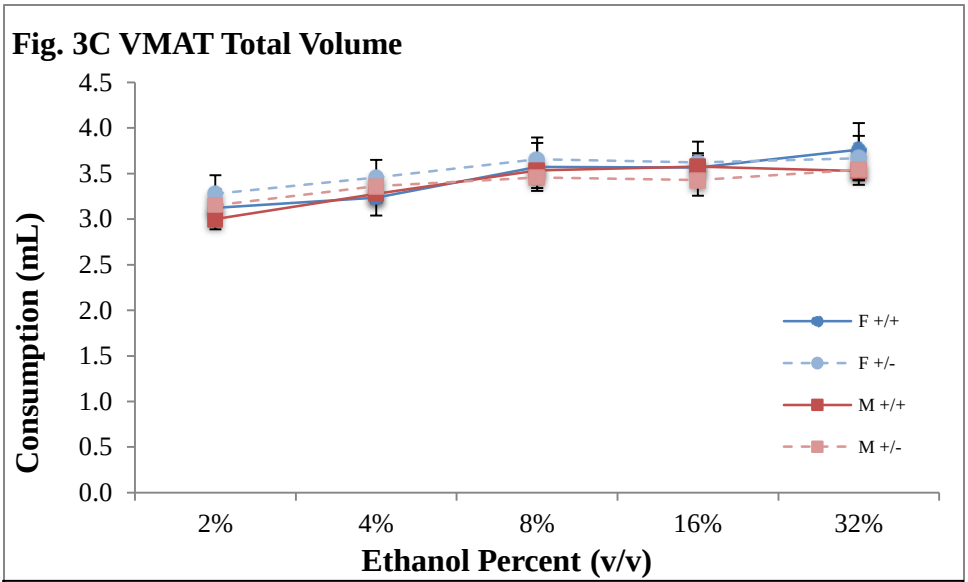


Figure 3C. Total fluid consumption increased slightly across the experiment. A significant effect of CONCENTRATION (F [4,36] = 12.2, p<0.0001) was observed.

For all groups, preference increased up to 8% ethanol, but then decreased dramatically at higher concentrations. Overall levels of preference were most similar to DAT BX and female DAT mice for 2%-8% solutions (50%, 60%, and 70% respectively), but preference was reduced to 40% for the 16%, in between levels of preference for DAT and DAT BX mice) ethanol solution and 20% for the 32% ethanol solution, which is similar to male DAT mice. The ANOVA for ethanol preference indeed showed a significant effect of CONCENTRATION (F [4,36] = 45.5, p<0.0001; Fig 3D).

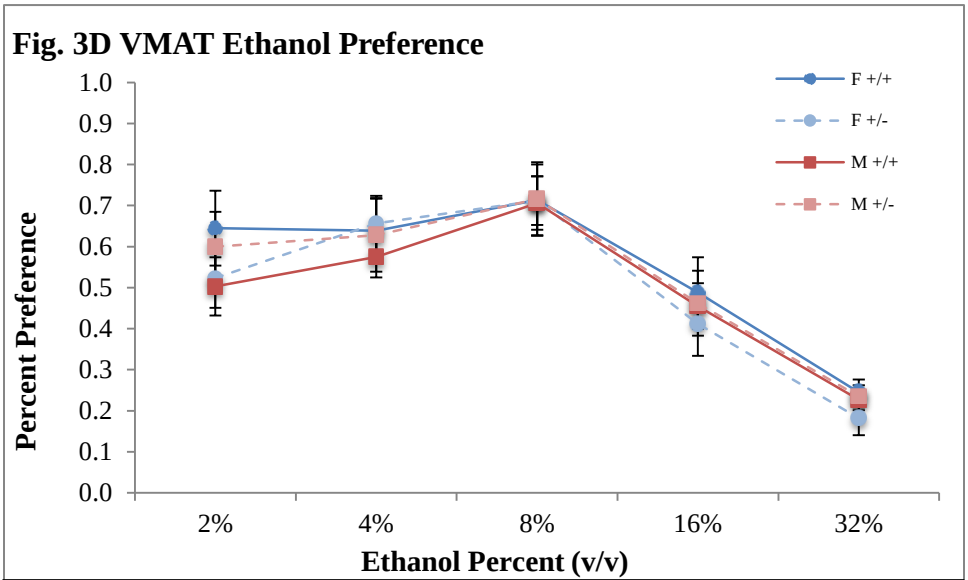


Figure 3D. Preference followed the standard pattern observed for other strains, and as such also showed a significant effect of CONCENTRATION ($F [4,36] = 45.5$, $p<0.0001$)

When ethanol consumption was represented as g/kg/day, a similar pattern of increased consumption with increased concentration of ethanol solution was observed, as with all other strains. This is suggestive of mice titrating the dose to some optimal individual level. Even though the mice showed a decrease in the volume of ethanol solutions consumed at the higher concentrations, their actual intake of ethanol increased across substantially across the initial ethanol concentrations and then largely stabilized at 8% and above, and *post hoc* analysis showed significant increases between all solutions except 16% and 32% ethanol solutions. The previous observation of overall increases in total fluid consumption is therefore more likely to be due to overall increased water consumption. The mice could have attempted to dilute their consumption of the higher concentrations of ethanol by drinking water concurrently, or the mice could have consumed more water to alleviate the effects of dehydration that might arise with increased ethanol consumption. Analysis revealed a significant effect of CONCENTRATION ($F [4,36] = 421.8$, $p<0.0001$; Fig 3E).

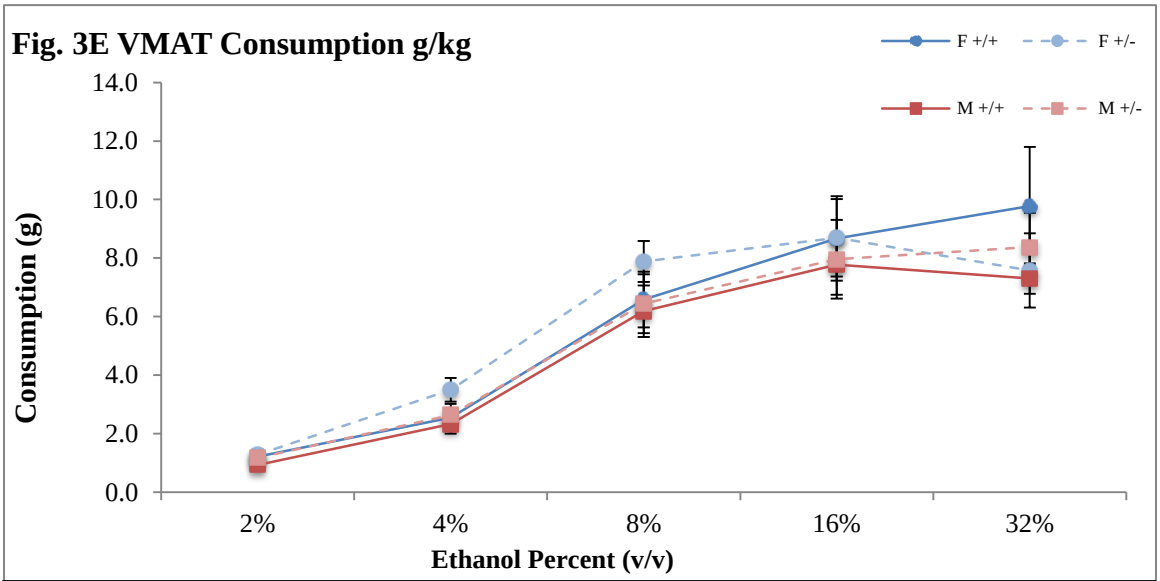


Figure 3E. Consumption increased with ethanol solution concentration, and there was a significant effect of CONCENTRATION (F [4,36] = 421.8, p<0.0001).

Experiment 1D: VMAT BX

An overall effect of genotype was observed for ethanol solution consumption, ethanol preference, and ethanol consumption in g/kg/day. However, the effect was the opposite of the original hypothesis—WT mice consumed more and had higher preference for ethanol than heterozygous congenic VMAT KO mice. VMAT BX mice, except male hKO mice, showed similar levels of ethanol solution consumption and preference to DAT BX mice, and VMAT BX male hKO mice showed reduced levels of consumption and preference compared with VMAT BX WT mice and female mice of both genotypes. A sex effect was observed in VMAT BX mice where females consumed more ethanol (g/kg/day) and had higher ethanol preferences, which supports the original hypothesis. Finally, WT congenic VMAT BX mice showed higher consumption and preference than mixed VMAT mice, which is also consistent with the original hypothesis that any observed effect would be magnified in the congenic strain.

ANOVA for the volume of ethanol solution consumed revealed effects of GENOTYPE (F [1,36] F=9.7 , p<0.004; Fig 4A), CONCENTRATION (F [4,36] = 24.8, p<0.0001; Fig 4A) and a significant CONCENTRATION x SEX interaction (F [4, 36] = 3.0, p<0.02; Fig 4A). Although there was a significant overall effect of GENOTYPE in the ANOVA, and no significant GENOTYPE x CONCENTRATION interaction, genotypic differences were observed primarily for the lower concentrations of ethanol. Heterozygous KO mice drank less than their WT littermates, but these differences lessened as the concentration of ethanol increased, and the groups showed similar amounts of consumption for the highest ethanol concentration (as discussed below, these observations were confirmed in further *post hoc* analyses). For lower ethanol concentrations, males and females of the same genotype showed similar levels of consumption, however at higher concentrations females consumed more ethanol solution compared to males of the same genotype.

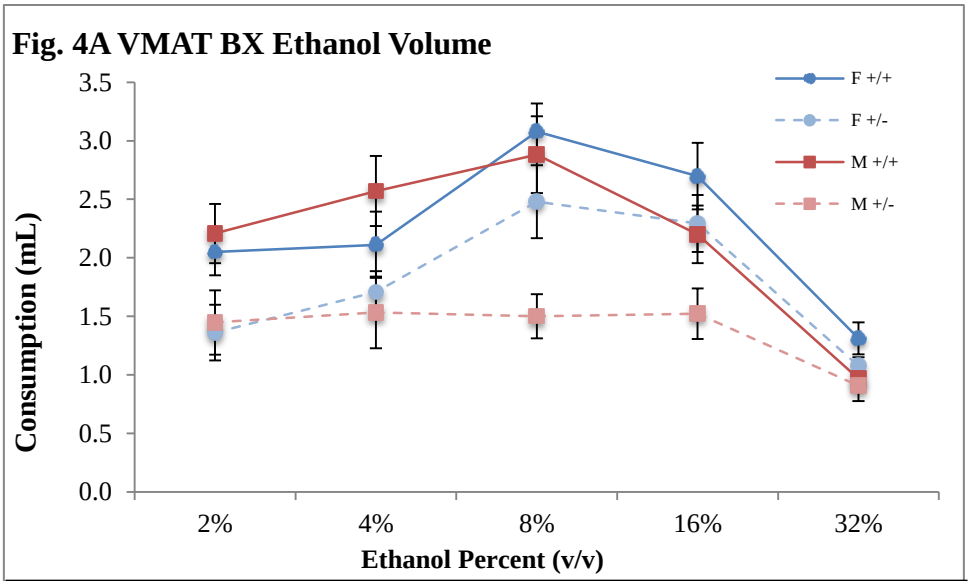


Figure 4A. Consumption of ethanol solution followed the overall pattern seen in other strains, and WT mice consumed more than +/- mice. Significant effects of GENOTYPE (F [1,36] F=9.7 , p<0.004) and CONCENTRATION (F [4,36] = 24.8, p<0.0001), and a significant CONCENTRATION x SEX interaction (F [4, 36] = 3.0, p<0.02) was observed.

Water volume consumption was inversely related to ethanol consumption, as before, and

analysis showed a significant effect of CONCENTRATION ($F [4,36] = 24.3$, $p<0.0001$; Fig 4B) and a significant CONCENTRATION x SEX interaction ($F[4, 36] = 3.3$, $p<0.01$).

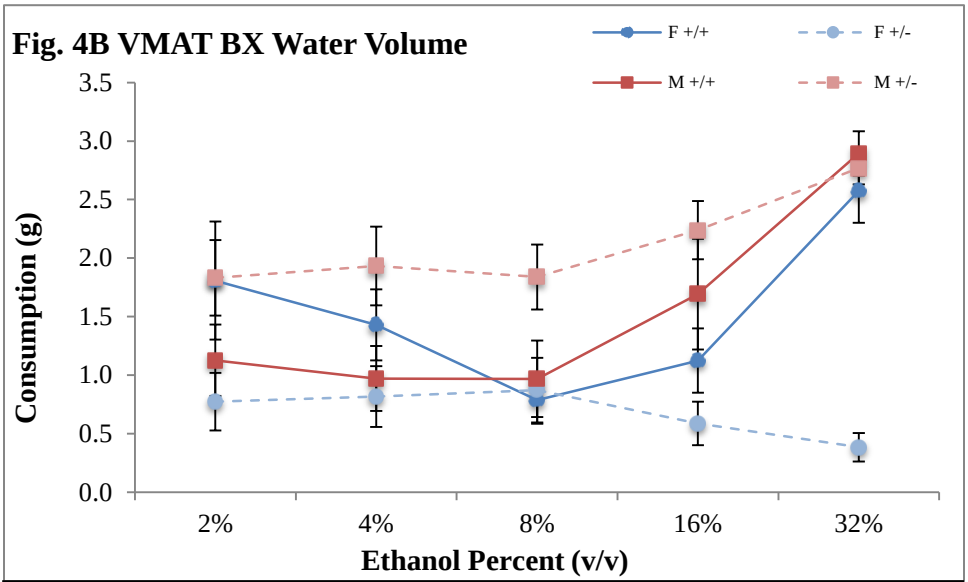


Figure 4B. WT VMAT BX mice showed an overall pattern of water consumption consistent with observations in other strains. M +/- mice showed elevated water consumption, and F +/- showed decreased water consumption. A significant effect of CONCENTRATION ($F [4,36] = 24.3$, $p<0.0001$) and interaction of CONCENTRATION x SEX ($F[4, 36] = 3.3$, $p<0.01$) were observed.

Total consumption showed a similar increase with ethanol concentration to that observed in other strains, supported by a significant effect of CONCENTRATION in the ANOVA ($F [4,36] = 6.3$, $p<0.0001$; Fig 4C).

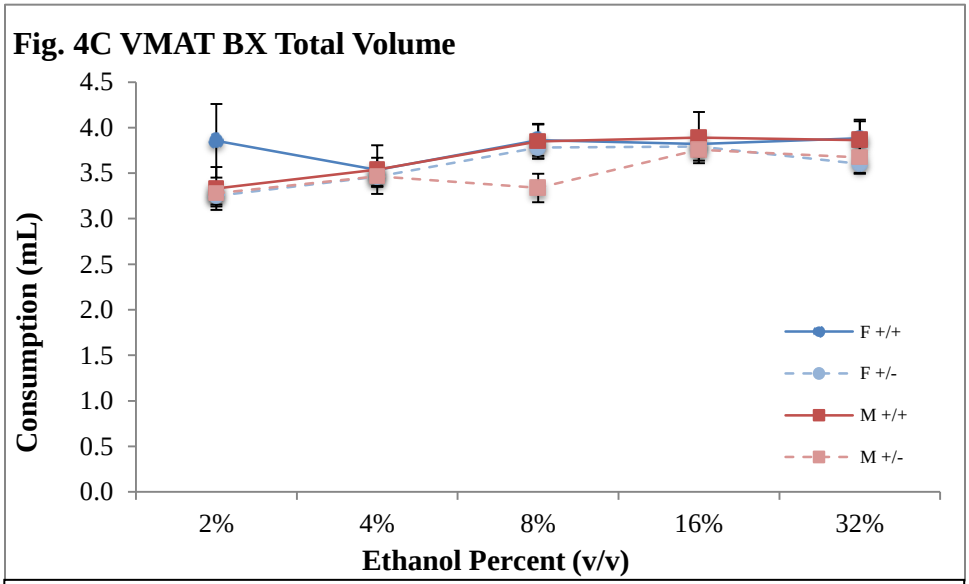


Figure 4C. Total consumption increased with ethanol solution concentration, represented by an effect of CONCENTRATION ($F [4,36] = 6.3$, $p<0.0001$).

Given the patterns for consumption ethanol solutions, water, and total fluid volume, it is not surprising that significant effects of GENOTYPE ($F_{1,36}$ F= 8.5, $p<0.0059$; Fig 4D), CONCENTRATION ($F_{4,36} = 26.6$, $p<0.0001$) and CONCENTRATION x SEX ($F_{4, 36} = 3.0$, $p<0.02$) were seen in the ANOVA for ethanol preference. Preference for ethanol increased from low to moderate ethanol concentrations, and then decreased again for the higher ethanol concentrations for all groups. hKO mice showed reduced overall preference for the lower concentrations of ethanol compared to WT mice, which had strong preferences (approximately 70% for 8% ethanol). Female hKO mice showed similar preferences to WT mice at the lowest concentrations, but greater preferences at middle concentrations. At the highest concentrations all mice, of both sexes and genotypes, showed reduced preference for ethanol solutions. The levels of preference observed were similar to the other congenic strain, DAT BX, and VMAT BX WT mice showed higher preference for all concentrations than VMAT mice. VMAT BX hKO mice showed slightly lower preference for 2%-16% ethanol solutions, but increased preference for 32% ethanol solution (VMAT 20%, VMAT BX 25%).

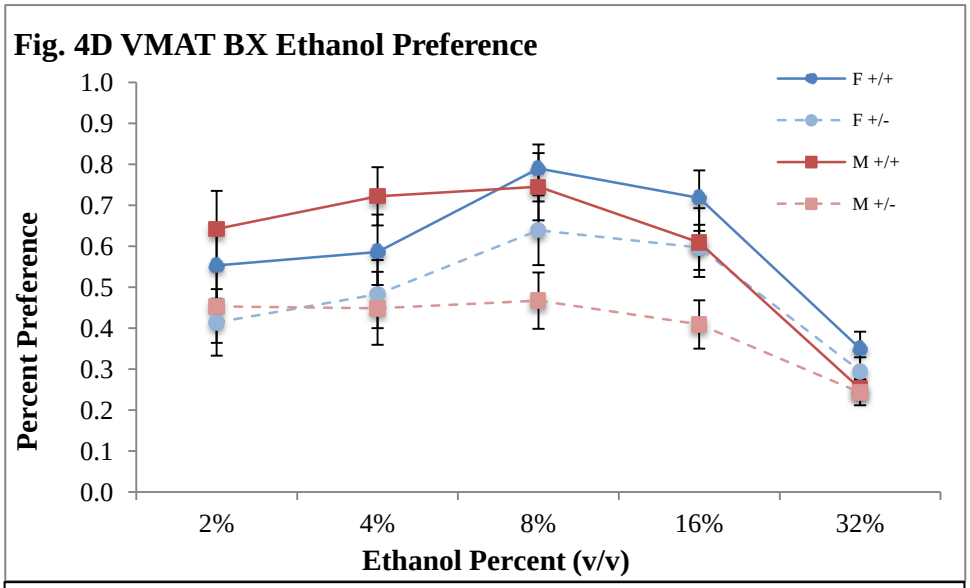


Figure 4D. Ethanol preference followed a similar pattern to other strains, resulting in a somewhat U-shaped curve. Significant effects of GENOTYPE ($F_{1,36}$ F= 8.5, $p<0.0059$) and CONCENTRATION ($F_{4,36} = 26.6$, $p<0.0001$), and an interaction of CONCENTRATION x SEX ($F_{4, 36} = 3.0$, $p<0.02$) was observed.

When ethanol consumption was calculated as g/kg/day, significant effects of GENOTYPE ($F [1,36] = 7.48$, $p<0.009$; Fig 4E), SEX ($F [1, 36] = 44.9$, $p<0.0001$), CONCENTRATION ($F [4,36] = 141.0$, $p<0.0001$), and a significant CONCENTRATION x SEX interaction ($F [4, 36] = 17.5$, $p<0.0001$. 18.3, $p<0.0001$) were observed in the ANOVA. Consumption increased substantially for all VMAT BX mice as the ethanol concentration increased, and this effect was much greater for female mice of both genotypes. KO mice showed decreased ethanol consumption from their WT counterparts for moderate to high ethanol concentrations. As expected, female mice drank more than male mice. This effect was most prominent at moderate to high ethanol concentrations. As far as genotype, WT mice drank more than hKO mice, and females drank more than males. When compared with VMAT mice, apart from significant effects of GENOTYPE and SEX for VMAT BX mice not seen with VMAT mice, VMAT BX females consumed twice as much as VMAT mice at 16% and 32% (8 grams for VMAT, 16 grams for VMAT BX females) ethanol solution. VMAT BX WT males consumed approximately the same amount as VMAT mice for all concentrations, and VMAT BX hKO males showed slightly decreased consumption at the lower concentrations, but arrived as similar levels of consumption to both VMAT BX WT males and VMAT mice at 16% and 32% ethanol solutions.

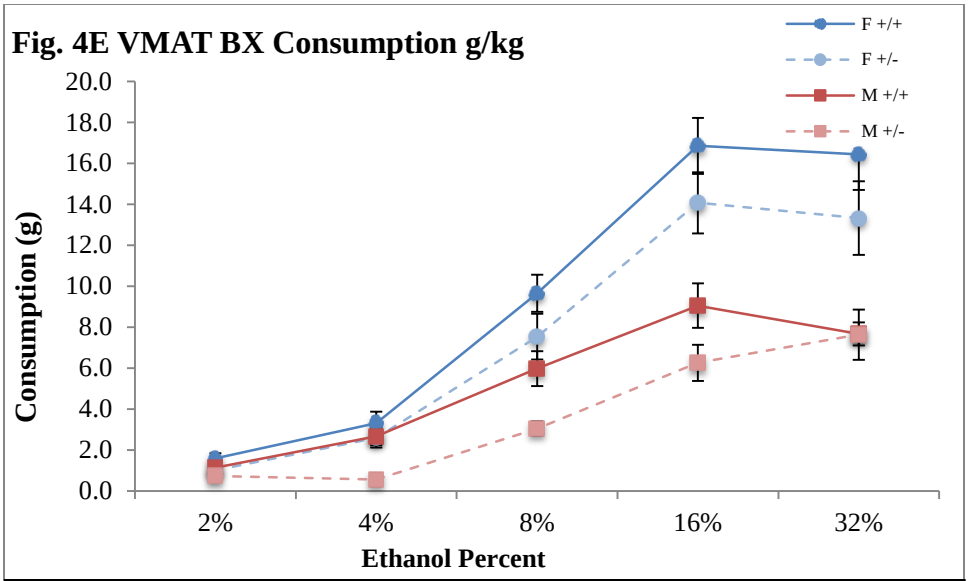


Figure 8E. Consumption increased for all groups, but female WT mice showed consistently higher levels of consumption. Significant effects of GENOTYPE (F [1,36] = 7.48, p<0.009; Fig 4E) and SEX (F [1, 36] = 44.9, p<0.0001), CONCENTRATION (F [4,36] = 141.0, p<0.0001), and a significant CONCENTRATION x SEX (F [4, 36] = 17.5, p<0.0001. 18.3, p<0.0001) interaction were observed.

Because of the significant effects observed in the original ANOVA, subsequent ANOVAs were preformed separately for each sex. For females, analysis of ethanol solution consumption revealed a significant effect of CONCENTRATION (F [4,18] = 22.7, p<0.0001), but this *post hoc* ANOVA did not find a significant effect of GENOTYPE (p>0.05). Consumption significantly increased between 2% and 8% ethanol solution (p<0.0001), and significantly decreased between 8% and 32% ethanol solution (p<0.0001), which describes the inverted U-shaped curve observed when ethanol solution consumption was initially analyzed. No significant effects or interactions were observed between SEX or GENOTYPE with total fluid consumption in the initial ANOVA, so *post hoc* analyses are not presented for this measure. Highest ethanol preference was observed at the 8% solution (80% for WT, 65% for hKO), and preference from 2%-8% solution increased and decreased from 8%-32%. *Post hoc* analysis confirmed an effect of CONCENTRATION (F [4,18] = 20.1, p<0.0001) and determined there

was significant increase in preference between 2% and 8% ethanol solution ($p<0.0001$), and significant decrease for 8% and 32% solutions ($p<0.0001$). No significant effect of GENOTYPE was observed for ethanol preference. Finally, there was an effect of CONCENTRATION ($F [4,18] = 100.6$, $p<0.0001$) on consumption as g/kg/day. Moreover, there were significant increases, determined by *post hoc* means comparisons (independent of genotype), between the 2% solution and 8%-32% solutions ($p<0.0001$ for all).

The same analysis was performed for males, after identifying significant effects and/or interactions involving SEX or GENOTYPE in the initial ANOVA. When ethanol solution consumption was subjected to *post hoc* analyses, significant effects of GENOTYPE ($F [1,18] = 8.914$, $p<0.0079$) and CONCENTRATION ($F [4,18] = 8.4$, $p<0.0001$) were observed. Additionally, a significant increase in ethanol solution consumption was observed between the 2% and 8% solutions ($p<0.0001$), and a significant decrease between the 8% and 32% solutions ($p<0.0001$), by *post hoc* means comparisons. Water consumption showed an effect of CONCENTRATION ($F [4,18] = 13.2$, $p<0.0001$), and significant increases were observed in water consumption between 2% and 8% ($p<0.0005$), 16% ($p<0.02$), and 32% ($p<0.002$) solutions, determined by *post hoc* means comparisons, independent of genotype. Again, total fluid consumption was not included in *post hoc* tests because there was no effect or interaction between SEX or GENOTYPE and total fluid consumption in the initial ANOVA. Analysis of ethanol preference revealed significant effects of GENOTYPE ($F [1,18] = 7.7$, $p<0.02$) and CONCENTRATION ($F [4,18] = 11.4$, $p<0.0001$). This represents the increased preference in WT males compared with hKO males. Significant increases in preference were observed between the 2% and 8% solutions ($p<0.0001$), and significant decreases were observed between the 8% (maximum preference was observed at this concentration, 75% for WT and 45% for hKO

mice) and 32% solutions ($p<0.0001$), as determined by *post hoc* means comparisons. Finally, analysis of ethanol consumption as g/kg/day showed effects of GENOTYPE [$F [1,18] = 8.3$, $p<0.01$) and CONCENTRATION ($F [4,18] = 41.4$, $p<0.0001$). Significant increase in consumption was observed between the 2% and all other solutions ($p<0.0001$ for all effects). For this measure, *post hoc* ANOVA confirmed a significant effect of GENOTYPE ($p<0.01$).

After observing a significant effect of GENOTYPE in analysis of male VMAT BX data, analyses were further split by GENOTYPE in subsequent ANOVAs. For male WT mice, all previous significant effects were confirmed. For ethanol solution consumption, there was an effect of CONCENTRATION ($F [4,9] = 7.1$, $p<0.0005$), and *post hoc* analysis showed a significant decrease in ethanol solution consumption from 8% to 16% and 32% solutions ($p<0.0001$ for both). Water consumption increased across the experiment, and there was a significant effect of CONCENTRATION ($F [4,9] = 9.4$, $p<0.0001$), and this increase was significant for 2%, 4%, 8% ($p<0.0001$ for all), and 16% ($p<0.004$) ethanol solutions compared with 32% solution. Analysis of total fluid consumption was also consistent with previous analyses, and a significant effect of CONCENTRATION ($F [4,9] = 5.9$, $p<0.001$) was observed. *Post hoc* analysis showed significant increases between 2% solution and 8% ($p<0.0001$), 16% ($p<0.0005$), and 32% ($p<0.0008$) solutions. Ethanol preference was also significantly affected by CONCENTRATION ($F [4,9 = 9.5$, $p<0.0001$), and significant decreases in preference were observed between solutions 8% and 16-32% ($p<0.0001$) via *post hoc* means comparisons. Finally, the previously identified increase in g/kg/day ethanol consumption was confirmed by a significant effect of CONCENTRATION ($F [4,9] = 22.9$, $p<0.0001$), with significant increases in consumption observed between the 2% solution and 8-32% solutions ($p<0.0001$ for all).

Analysis of male hKO data showed no significant effect of CONCENTRATION on ethanol solution consumption ($p>0.05$). A significant increase in water consumption was observed, confirmed with an observed significant effect of CONCENTRATION ($F [4,9] = 4.0$, $p<0.009$) and *post hoc* means comparison, independent of genotype, showed significant increase between 2% and 32% solutions ($p<0.002$). Total fluid consumption was affected by CONCENTRATION ($F [4,9] = 5.0$, $p<0.003$), and significant increases were observed between 2%, and the 16% ($p<0.0009$) and 32% ($p<0.005$) solutions, calculated by *post hoc* means comparisons, independent of genotype. An effect of CONCENTRATION ($F [4,9] = 2.7$, $p<0.05$) was observed when ethanol preference data was analyzed, and *post hoc* means comparison showed a significant decrease in preference between 8% and 32% ($p<0.009$) ethanol solutions. Finally, the increases in g/kg/day consumption previously described were seen in this analysis as well, described by a significant effect of CONCENTRATION ($F [4,9] = 20.2$, $p<0.0001$), and significant increases were observed between 2% and 8-32% solutions ($p<0.02$, $p<0.0001$, $p<0.0001$ respectively).

Given the significant effects involving GENOTYPE in the initial ANOVA, means were compared for each concentration to determine the nature of the genotypic effects. When ethanol solution consumption was analyzed, WT mice consumed more ethanol solution at 2%-16% ($p<0.04$) concentrations than hKO, significant by *post hoc* means comparisons. When ethanol preference was analyzed, WT mice showed higher preference for at 4%-16% ($p<0.04$) concentrations than hKO, significant by *post hoc* means comparisons. Lastly, when consumption as g/kg was analyzed, WT mice consumed more ethanol solution at 2%, 8%, and 16% ($p<0.03$) concentrations than hKO, significant by *post hoc* means comparisons.

Experiment 2A: DAT KO

Results from the escalation study support the original hypothesis that mice will voluntarily escalate their ethanol consumption when presented with 8% ethanol solution 2 times per week. Females had overall higher levels of consumption as measured by volume and g/kg/day. However, when escalation was measured as individual change from the group mean of day 1, males showed much higher levels of escalation because they started at slightly lower levels of consumption. However, all groups escalated their ethanol consumption and showed positive increases as measured by percent change in consumption. All groups showed increased consumption of ethanol across sessions, confirmed by a significant effect of SESSION in the ANOVA ($F [5,44] = 12.7$, $p<0.0001$; Fig 5A). Females consumed a greater volume of ethanol than males for the first and final sessions, but consumption equalized during the middle sessions, resulting in significant SESSION x SEX interaction ($F [5,44] = 2.9$, $p<0.018$).

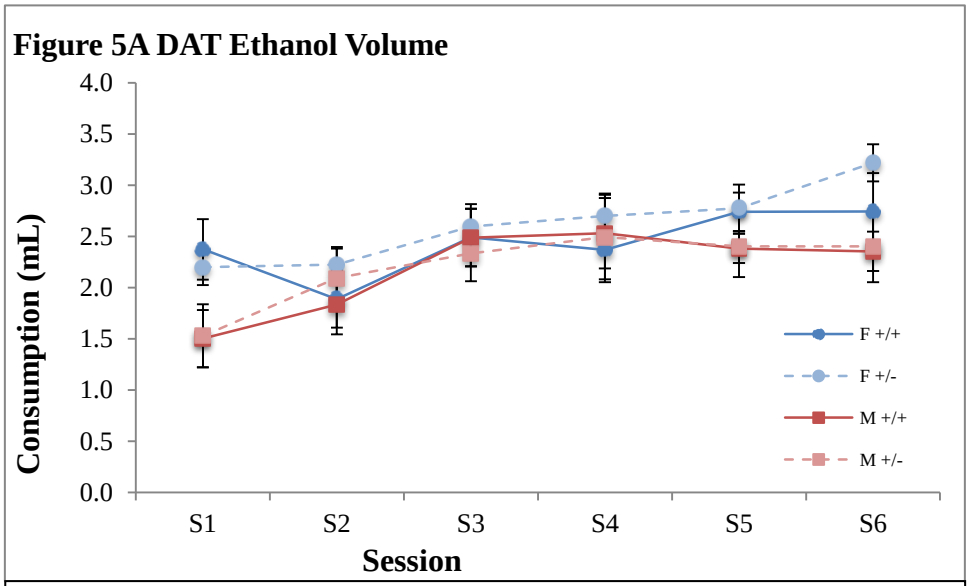


Figure 5A. All groups increased their consumption of ethanol solution across the experiment, and analysis revealed a significant effect of SESSION ($F [5,44] = 12.7$, $p<0.0001$) and interaction of SESSION x SEX ($F [5,44] = 2.9$, $p<0.018$), reflecting increased ethanol consumption in females, particularly in the first and final sessions.

All groups decreased their water consumption across sessions, corroborated by a significant effect of SESSION in the ANOVA ($F [5,44] = 5.6$, $p<0.0001$; Fig 5B), and consistent with previous observations that the pattern for water consumption was generally opposite to that for ethanol. Male WT mice initially showed higher levels of water consumption, but decreased water consumption over sessions to similar amounts of the other groups ($p<0.0001$ between session 1 and 3-6 by *post hoc* comparison of means independent of genotype). By the final session, M WT and F hKO mice were drinking similar levels of water, but these levels were lower than F WT and M hKO mice. Thus, significant interactions of SESSION x GENOTYPE ($F [5,44] = 2.5$, $p<0.028$) and SESSION x GENOTYPE x SEX ($F [5,44] = 2.3$, $p<0.045$) were observed.

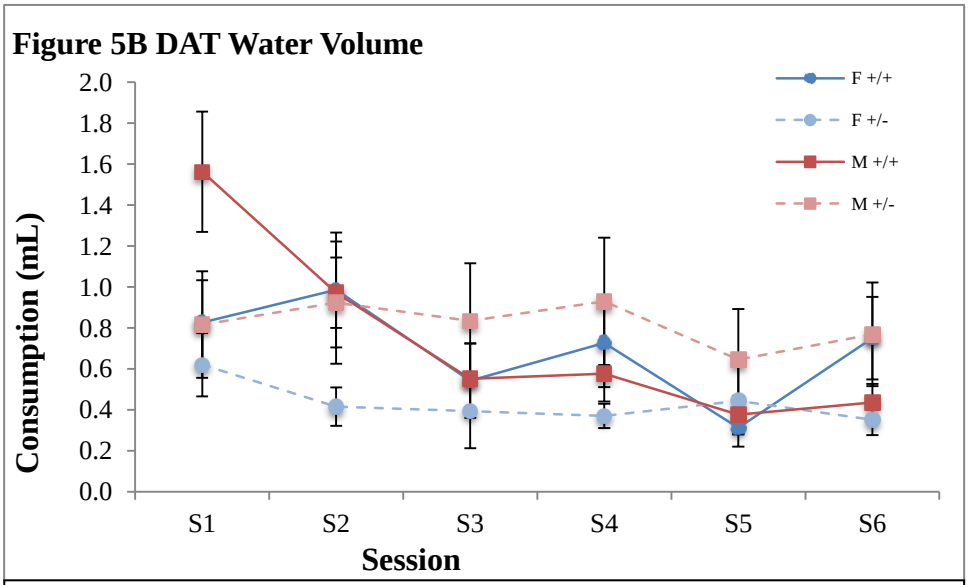


Figure 5B. Water consumption decreased for all groups, and male WT mice showed elevated initial water consumption that decreased to levels similar to other groups. A significant effect of SESSION ($F [5,44] = 5.6$, $p<0.0001$), and significant interactions of SESSION x GENOTYPE ($F [5,44] = 2.5$, $p<0.028$) and SESSION x GENOTYPE x SEX ($F [5,44] = 2.3$, $p<0.045$) were observed.

Total fluid consumption for female and male hKO mice showed similar patterns of increased total fluid consumption across the sessions, while females started and ended at higher levels. Male WT fluctuated around 3 mL of consumption for the duration of the experiment. hKO mice started at slightly lower levels of fluid consumption, and ended at slightly higher levels of fluid consumption. Analysis showed that total fluid consumption was significantly affected by SESSION ($F [5,44] = 6.2$, $p<0.0001$; Fig 5C), and significant interactions of SESSION x GENOTYPE ($F [5,44] = 3.3$, $p<0.007$) and SESSION x SEX ($F [5,44] = 4.8$, $p<0.0004$) were observed.

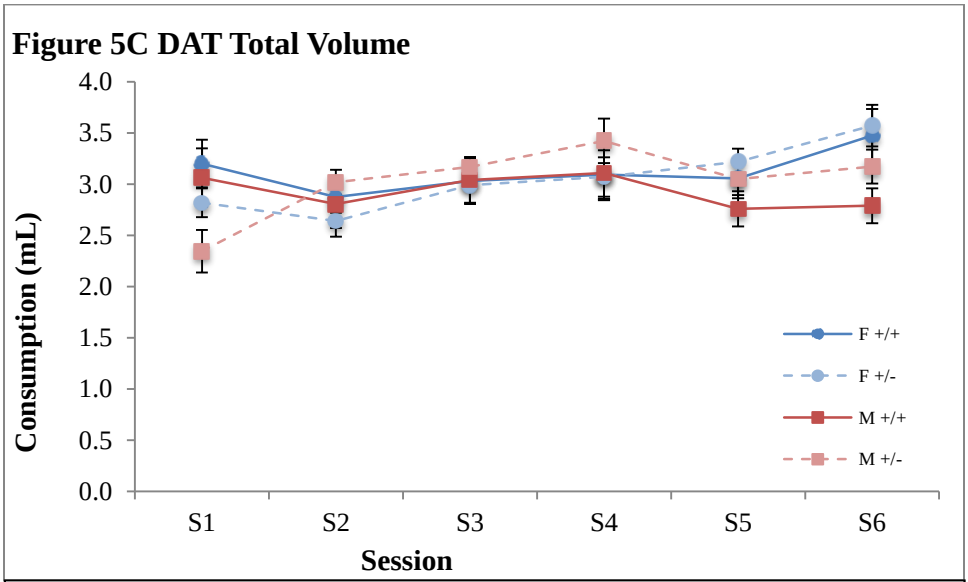


Figure 5C. Total levels of fluid consumption increased, females overall consumed more total fluid, and male WT mice consumed a consistent about of fluid. These observations are supported by a significant effect SESSION ($F [5,44] = 6.2$, $p<0.0001$; Fig 5C), and significant interactions of SESSION x GENOTYPE ($F [5,44] = 3.3$, $p<0.007$) and SESSION x SEX ($F [5,44] = 4.8$, $p<0.0004$).

Given the previously mentioned differences in consumption between males and females, it is not surprising that similar differences were observed when ethanol preference was analyzed. Females started at much higher levels of preference (around 75%) compared to males (around 60%). However, they escalated preference to similar levels (around 80%, except female hKO

that showed 90% preference). These results were confirmed by a significant effect of SESSION (F [5,44] = 8.5, p<0.0001) in the ANOVA.

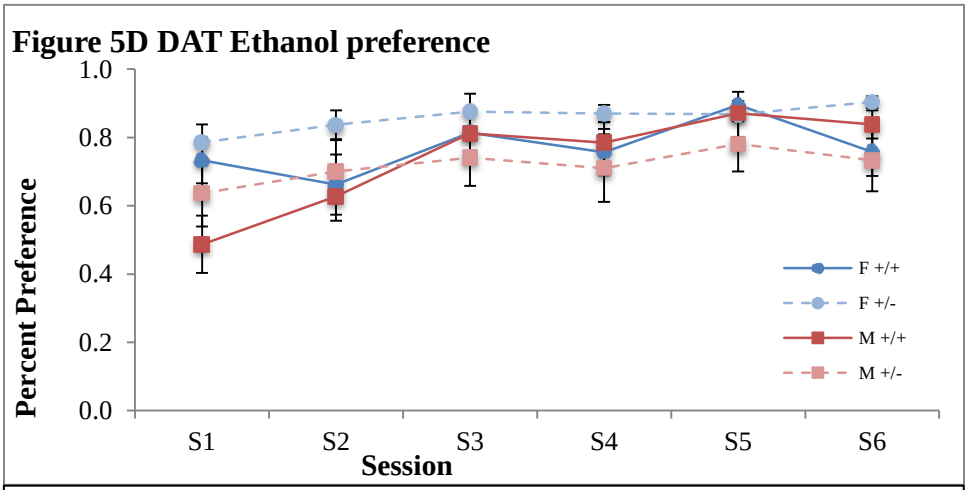


Figure 5D. While females showed initial higher preference than males initially, all groups increased preference to show generally similar high levels of preference by the final session, and a significant effect of SESSION (F [5,44] = 8.5, p<0.0001) was observed.

When consumption was analyzed as g/kg/day, females showed elevated initial levels of consumption (8 g/kg) compared with males (4 g/kg). Both males and females increased ethanol consumption by about 2.25 mL from the first to final session. This pattern of results was confirmed by a significant by an effect of SEX (F [1,44] = 19.0, p<0.0001; Fig 5E) and SESSION (F [5,44] = 13.8, p<0.0001), and a significant SESSION x SEX interaction (F [5,44] = 3.0, p<0.01).

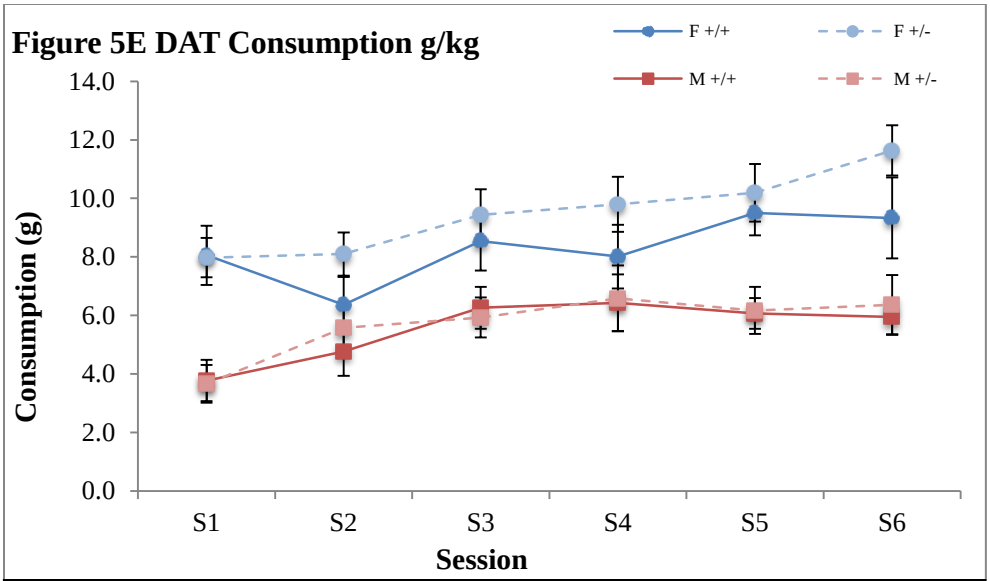


Figure 5E. Females showed higher levels of consumption, SEX (F [1,44] = 19.0, p<0.0001) and SESSION (F [5,44] = 13.8, p<0.0001), and an interaction of SESSION x SEX (F [5,44] = 3.0, p<0.01).

All of the previous measures showed results consistent with the first experiment—females consumed more than males, regardless of genotype. While the total amount of ethanol consumed did not differ between genotypes, the rate of escalation was analyzed to investigate another aspect of escalation via percent change from the baseline (day 1). While for all of the other measures females showed higher levels of consumption, when escalation was considered in terms of percent change, males showed a higher percent change than females starting with session 2, in part due to initially lower levels of consumption in males. Males therefore escalated at a faster rate than females and to a somewhat greater extent. Analysis of the data showed significant effects of SEX (F [1,44] = 7.2, p<0.01; Fig 5F) and SESSION (F [5,44] = 11.0, p<0.0001), as well as a significant SESSION x SEX interaction (F [5,44] = 2.9, p<0.02).

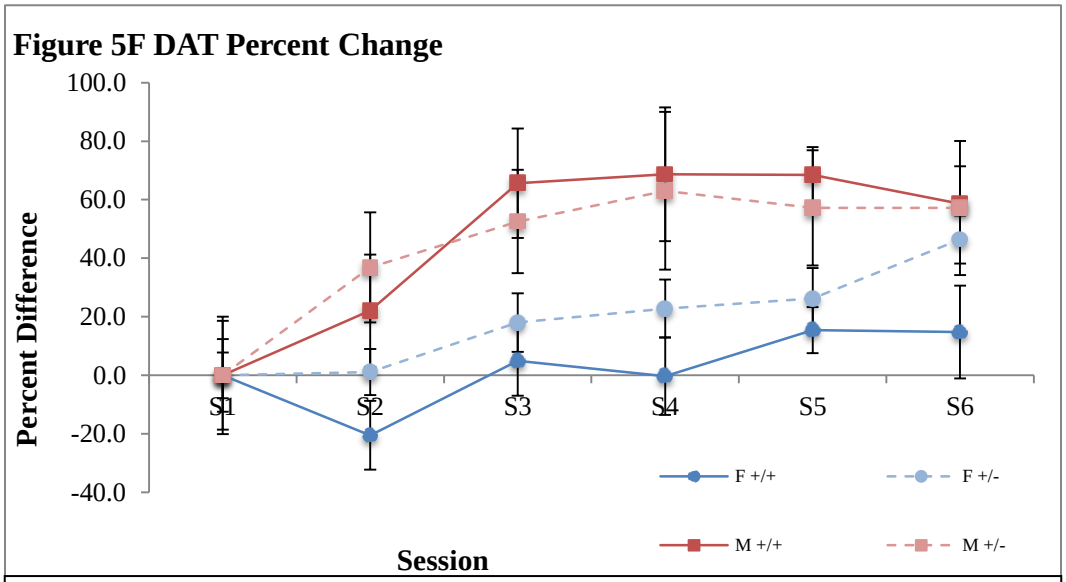


Figure 5F. Males escalated at a faster rate than females, and overall escalated to a greater extent than females. Significant effects of SEX ($F [1,44] = 7.2$, $p<0.01$; Fig 5F) and SESSION ($F [5,44] = 11.0$, $p<0.0001$) and a significant interaction of SESSION x SEX ($f [5,44] = 2.9$, $p<0.02$) was observed.

Due to the significant effects of sex in the initial ANOVA, separate ANOVA were performed for males and females. When data from females was analyzed separately, previously observed effects of SESSION were confirmed—increased ethanol consumption, increased fluid consumption, increased ethanol preference, and positive percent change. The only significant effect involving GENOTYPE was with water consumption, which is discussed subsequently. Significant increases in ethanol solution consumption were confirmed by the observed significant effect of SESSION ($F [5,22] =12.0$, $p<0.0001$). Decreased water consumption over the course of the experiment was confirmed by a significant effect of SESSION ($F [5,22] = 3.7$, $p<0.04$). Female WT consumed more water each session than female hKO mice, but these differences were significant only for sessions 2, 4, and 6. This is supported by a significant SESSION x GENOTYPE interaction ($F [5,22] = 2.5$, $p<0.04$). All females increased their total fluid consumption across sessions, and total fluid consumption was significantly affected by SESSION ($F [5,22] = 9.7$, $p<0.0001$). There were no significant effects on ethanol preference in

the initial ANOVA, so there was no further analysis. Again, ANOVA confirmed the previously observed increase in ethanol consumption expressed as g/kg/day, showing a significant effect of SESSION ($F [5,22] = 11.8$, $p<0.0001$). Finally, females showed increase in percent change across the experiment, as shown by a significant effect of SESSION ($F [5,22] = 12.5$, $p<0.0001$) on the percent change measure. As previously mentioned, no significant effect of genotype was observed for any measure ($p>0.05$ for all analyses), and the only interaction involving genotype was greater water consumption in female WT mice.

Data from males revealed similar patterns overall; although no significant effects of GENOTYPE alone were observed, significant interactions between SESSION and GENOTYPE were observed for water consumption and total fluid consumption. All males increased ethanol solution consumption across sessions, as confirmed by a significant effect of SESSION ($F [5,22] = 6.2$, $p<0.0001$), from an average 1.5 mL ethanol solution consumption to 2.5 mL ethanol solution consumption when first and final sessions were compared. Water consumption levels decreased for male WT mice, but male hKO mice maintained steady levels of water consumption across sessions. ANOVA showed a significant effect of SESSION ($F [5,22] = 3.3$, $p<0.02$) and a significant SESSION x GENOTYPE interaction ($F [5,22] = 2.4$, $p<0.04$). Males also increased total fluid consumption, however, male hKO mice showed a slightly higher increase in fluid consumption. This pattern of effects was confirmed by a significant effect of SESSION ($F [5,22] = 3.2$, $p<0.001$) and a significant SESSION x GENOTYPE interaction ($F [5,22] = 3.7$, $p<0.04$). As ethanol preference was not significantly affected by sex in the initial ANOVA, the results from the ANOVA were analyzed separately. Ethanol consumption expressed as g/kg/day increased across sessions showed a significant effect of SESSION in male mice ($F [5,22] = 6.4$, $p<0.0001$). As previously mentioned, the increase in ethanol consumption in males was also

apparent in a larger percent change in ethanol consumption across sessions as shown by a significant effect of SESSION ($F [5,22] = 6.7$, $p<0.0001$) in the ANOVA.

Further *post hoc* analyses were performed for each experimental group by 1-way ANOVA. Female WT mice showed increased ethanol solution consumption, confirmed by an effect of SESSION ($F [5,13] = 5.2$, $p<0.0005$). Significant increases in ethanol solution consumption were observed between sessions 1 and 5-6 ($p<0.04$). Water consumption decreased across the sessions, and as a result there was a significant effect of SESSION ($F [5,13] = 3.4$, $p<0.009$), with a significant decrease observed between sessions 1 and 5 ($p<0.007$). Total fluid consumption increased with an effect of SESSION ($F [5,13] = 4.7$, $p<0.001$) and a significant increase observed between session 1 and 6 ($p<0.005$). Ethanol preference was somewhat variable for this group, but there was an overall increase in preference across sessions. A significant effect of SESSION ($F [5,13] = 3.6$, $p<0.006$) was observed, and *post hoc* means comparisons showed a significant increase in preference between session 1 and session 5 ($p<0.007$). Previous observations that g/kg/day consumption increased across sessions were confirmed in this analysis by a significant effect of SESSION ($F [5,13] = 5.1$, $p<0.0006$), and *post hoc* means comparisons showed significant increases in g/kg/day consumption between sessions 1 and 5-6 ($p<0.03$). Finally, the percent change from baseline (day 1) increased across sessions. A significant effect of SESSION ($F [5,13] = 5.2$, $p<0.0005$) was observed, and significant increases in percent change were seen between sessions 1 and 5-6 ($p<0.04$ and $p<0.02$ respectively).

Similar trends were seen in female hKO mice. Ethanol solution consumption increased across sessions, a significant effect of SESSION ($F [5,9] = 8.8$, $p<0.0001$) was observed, and *post hoc* analysis showed significant increases between session 1 and sessions 3-6 ($p<0.04$).

Water consumption did not change significantly in female hKO mice. Total fluid consumption increased as demonstrated by a significant effect of SESSION ($F [5,9] = 5.3$, $p<0.0007$) in the 1 way ANOVA, and further analysis showed that this increase was significantly different between sessions 1 and 6 ($p<0.005$). Ethanol preference for female hKO mice increased with session. A significant effect of SESSION ($F [5,9] = 2.5$, $p<0.05$) was observed in the ANOVA, and *post hoc* means comparisons showed a significant increase between sessions 1 and session 5 ($p<0.007$). There was likely a ceiling effect for this group, because preference rose to about 90% by the final session. Consumption expressed as g/kg/day also increased, starting at 7.5 g/kg/day and reaching 11 g/kg/day by session 6. This was a significant increase, as supported by a significant effect of SESSION ($F [5,9] = 8.9$, $p<0.0001$) and *post hoc* significance between session 1 and sessions 5-6 ($p<0.03$ and $p<0.02$). This group escalated a maximum of 45% by the final session. ANOVA demonstrated a significant effect of SESSION ($F [5,9] = 8.8$, $p<0.0001$), and the increases were significant between sessions 1 and sessions 3-6 ($p<0.04$).

Analysis of male WT data was consistent with previously reported results. Ethanol solution consumption increased from initial consumption of 1.5 g ending at 2.5 g average consumption. There was a significant effect of SESSION ($F [5,11] = 3.8$, $p<0.005$) in the 1 way ANOVA, and the increases were significant between sessions 1 and sessions 3-6 ($p<0.01$). Water consumption significantly decreased, starting at 1.5 mL and ending at 0.5 mL consumption. This effect was confirmed by a significant effect of SESSION ($F [5,11] = 3.5$, $p<0.008$) in the 1 way ANOVA, and the decreases from session 1 to all other sessions, 2-6 ($p<0.01$ for session 2, $p<0.0001$ for all other differences). Total fluid consumption fluctuated around 3 mL. There was an overall effect of SESSION ($F [5,11] = 3.1$, $p<0.02$) in the ANOVA, but no significant differences when individual means were compared in *post hoc* analyses. There

was a marked increase in preference, with initial preference at around 50%, and final preference at around 85%. Again, there is the potential for a ceiling effect with these results. This increase was confirmed in the ANOVA in terms of a significant effect of SESSION ($F [5,11] = 3.6$, $p<0.007$), and *post hoc* significant increases between session 1 and sessions 3-6 ($p<0.0001$). Consumption in terms of g/kg/day also increased with session, confirmed by a significant effect of SESSION ($F [5,11] = 13.1$, $p<0.004$). *Post hoc* comparisons of individual means showed these increases to be significant from session 1 to sessions 3-6 ($p<0.005$). There was a sharp increase in consumption measured as percent change from the first session seen between sessions 1 and2, with further increases in subsequent sessions. A 20% increase was seen for session 2, which jumped to 65% increase for sessions 3-6. This pattern was confirmed by a significant effect of SESSION ($F [5,11] = 3.8$, $p<0.005$) and significant *post hoc* increases from session 1 to sessions 3-6 ($p<0.007$).

Finally, male hKO mice increased ethanol consumption across sessions. The 1-way ANOVA showed a significant effect of SESSION ($F [5,11] = 2.6$, $p<0.04$), and *post hoc* means comparison showed significant increases between session 1 and sessions 3-6 ($p<0.02$). Water consumption was variable, but stayed around 0.8 mL for all sessions. There was a significant effect of SESSION ($F [5,11] = 3.5$, $p<0.008$), but no significant *post hoc* increases or decreases. Total fluid consumption increased with session, starting at 2.5 mL and increasing to 3.25 mL. There was a significant effect of SESSION ($F [5,11] = 3.1$, $p<0.02$) in the ANOVA, and *post hoc* means comparisons showed significant increases from session 1 to sessions 2-6 ($p<0.001$). Ethanol preference increased as well, starting at 63% and reaching a maximum at session 5 of 78%. This increase produced a significant effect of SESSION ($F [5,11] = 3.6$, $p<0.007$), but there were no significant increases in *post hoc* means comparisons of session 1 with subsequent

sessions. Consumption as g/kg/day increased with session, for session 1 from 3.5 g/kg/day to 6.3 g/kg/day by session 6. This increase was significant with an effect of SESSION ($F [5,11] = 2.8$, $p<0.03$), and *post hoc* comparison of individual means showed that the increase was significant from session 1 to sessions 2-6 ($p<0.04$). Lastly, male hKO also showed positive percent increase across sessions, reaching a maximum of 63% for session 4, and leveling off at 57% for sessions 5 and 6. This increase was confirmed by significant by an effect of SESSION ($F [5,11] = 2.6$, $p<0.04$), and *post hoc* comparison of individual means showed the increase to be significant between session 1 and sessions 3-6 ($p<0.02$).

Due to significant effects of GENOTYPE observed in the initial ANOVA, *post hoc* means comparisons were performed on water volume and total fluid consumption measures. When water consumption was analyzed, WT mice showed higher water consumption for the first session only ($p<0.05$). When total fluid consumption was analyzed, only the first session was found to be significantly different, where WT consumed more than hKO mice ($p<0.02$).

Experiment 2B: DAT BX

Overall levels of consumption, both initial and final, for the DAT BX strain were elevated when compared with the DAT KO strain. The pattern described for DAT mice in this second experiment was similar to that observed for DAT BX mice—overall ethanol solution consumption, preference, consumption as g/kg/day, and percent change increased with session. As observed with experiment 1, no effect of sex was observed for any measures with DAT BX mice, while sex was a prominent factor in DAT KO mice. A significant interaction involving genotype was observed for consumption expressed as g/kg/day, and an overall effect of

GENOTYPE, along with a significant interaction involving genotype, was observed when percent change was analyzed.

When data for the volume of ethanol consumed in the escalation experiments was analyzed, a significant effect of SESSION was observed ($F [5,40] = 22.5$, $p<0.0001$; Fig 6A). All groups increased ethanol consumption across the three-week period, confirmed with *post hoc* ANOVA and means comparisons.

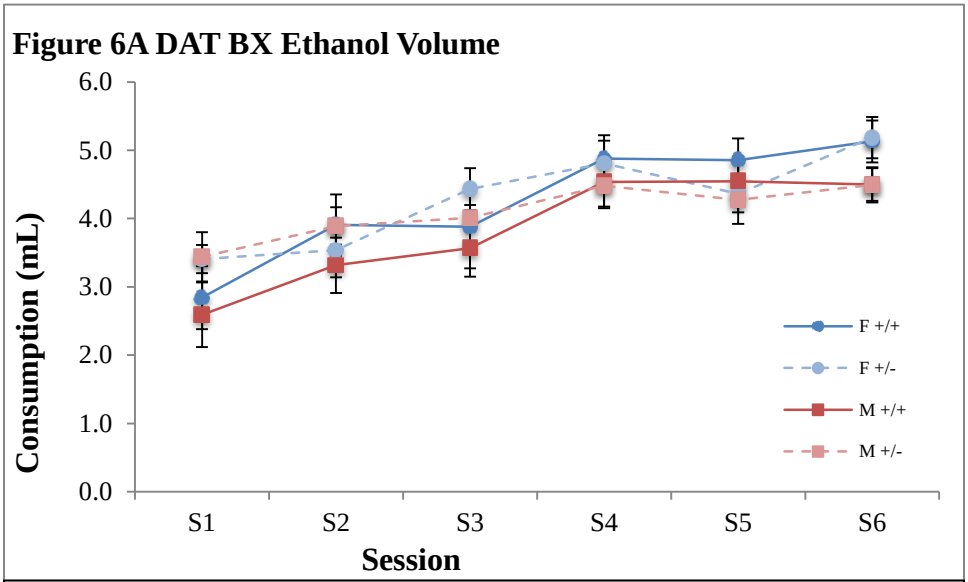


Figure 6A. Ethanol solution consumption increased for all DAT BX mice, and a significant effect of SESSION ($F [5,40] = 22.5$, $p<0.0001$) was observed.

Water volume consumed was quite variable for the first three sessions for all groups except female hKO mice, but approached steady lower levels for the final three sessions, and as such a significant effect of SESSION ($F [5,40] = 8.9$, $p<0.0001$; Fig 6B) was observed for water volume consumption.

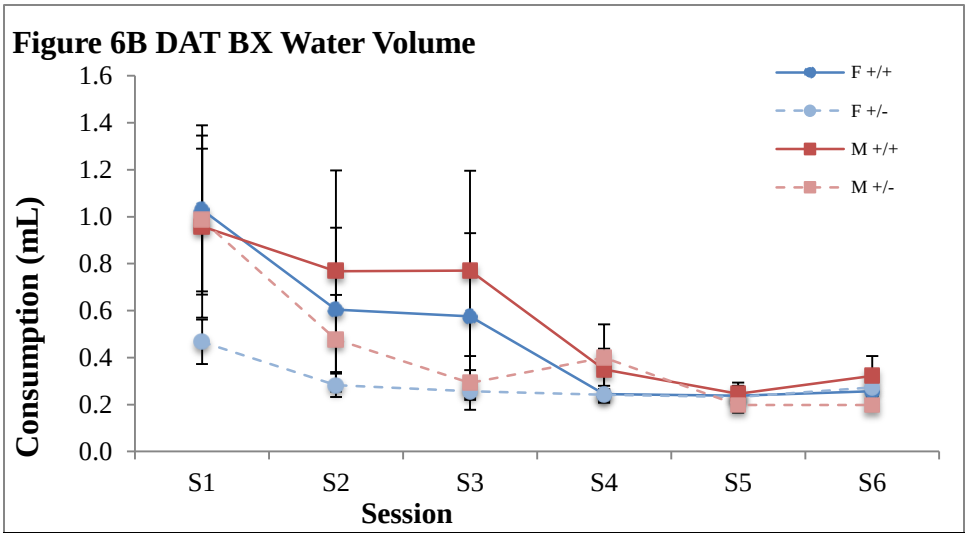


Figure 6B. While water consumption was highly variable for the initial sessions, it reached stable, lower levels by session 4. A significant effect of SESSION ($F [5,40] = 8.9$, $p<0.0001$) was observed.

Given the above results, it follows that an increase in total consumption was observed across the six experimental sessions, represented by a significant effect of SESSION in the ANOVA ($F [5,40] = 13.2$, $p<0.0001$; Fig 6C).

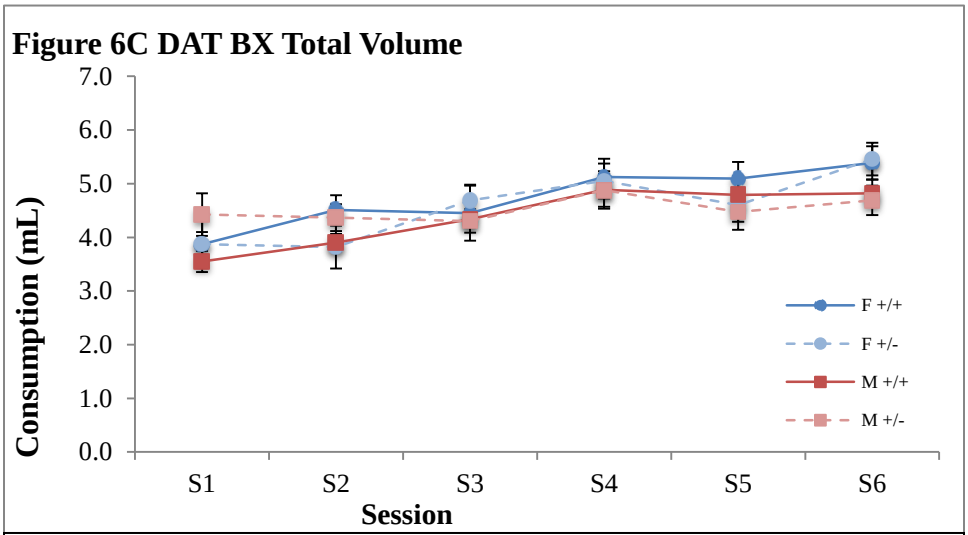


Figure 6C. Total fluid consumption increased with session, and a significant effect of SESSION ($F [5,40] = 13.2$, $p<0.0001$) was observed.

Changes in preference can be produced by changes in either consumption of ethanol solutions or water, both of which were changing here. As previously noted, all subject increased ethanol consumption and decreased water consumption across the sessions, so it follows that an increase in preference for ethanol was observed for all groups as confirmed by a significant effect of SESSION in the ANOVA ($F [5,40] = 9.8$, $p<0.0001$; Fig 6D).

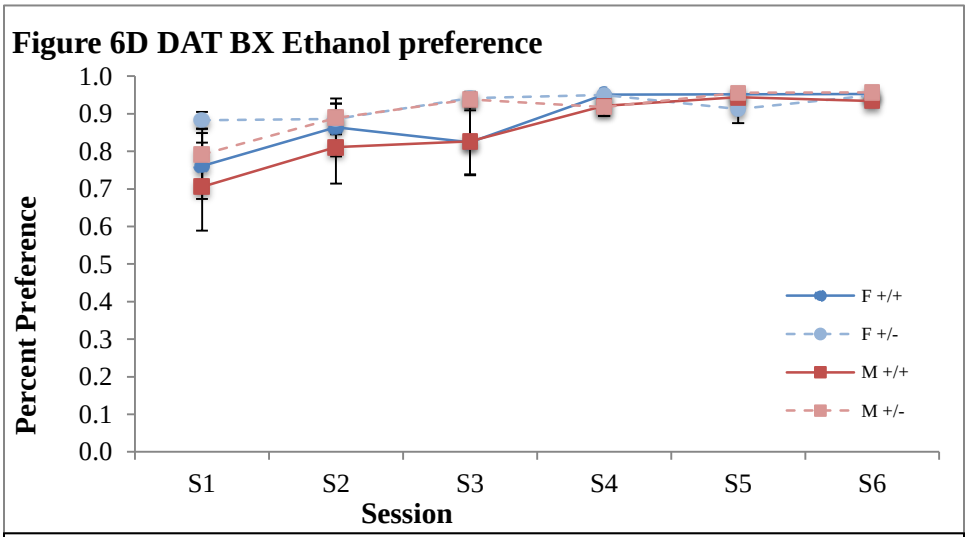


Figure 6D. Preference started at high levels, and increased to near maximal levels, where distinct ceiling effects were observed. This observation is confirmed by a significant effect of SESSION ($F [5,40] = 9.8$, $p<0.0001$) in the ANOVA.

When consumption was expressed as g/kg/day, all DAT BX mice increased their consumption. However, male hKO mice showed minimal increases in consumption, and consumed approximately 3 g/kg less than the other mice at the maximum levels of consumption, observed at session 6. As such, a significant effect of SESSION ($F [5,40] = 21.6$, $p<0.0001$; Fig 6E) and a significant SESSION x GENOTYPE interaction ($F [5,40] = 2.8$, $p<0.02$) were observed.

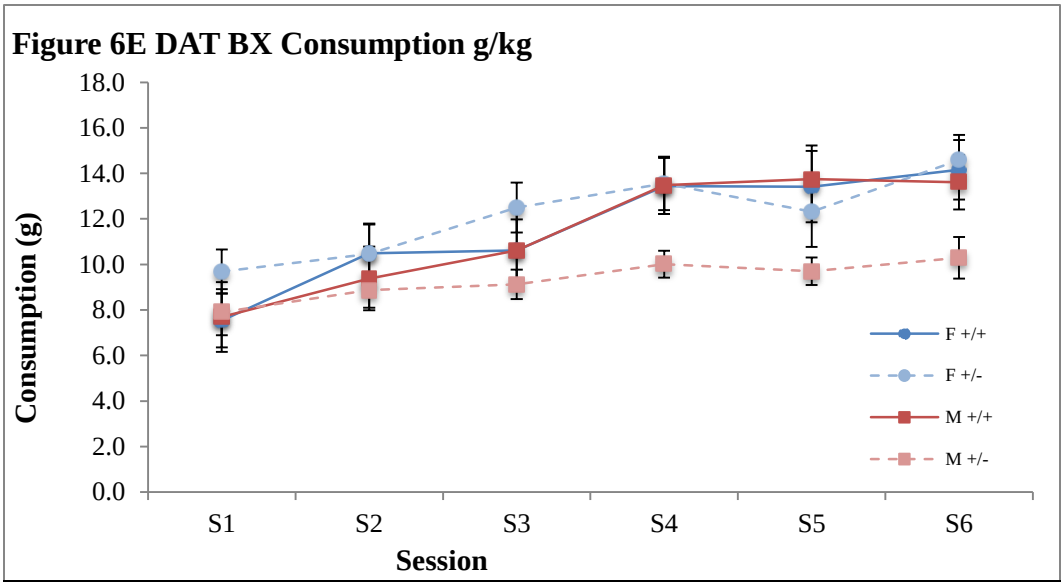
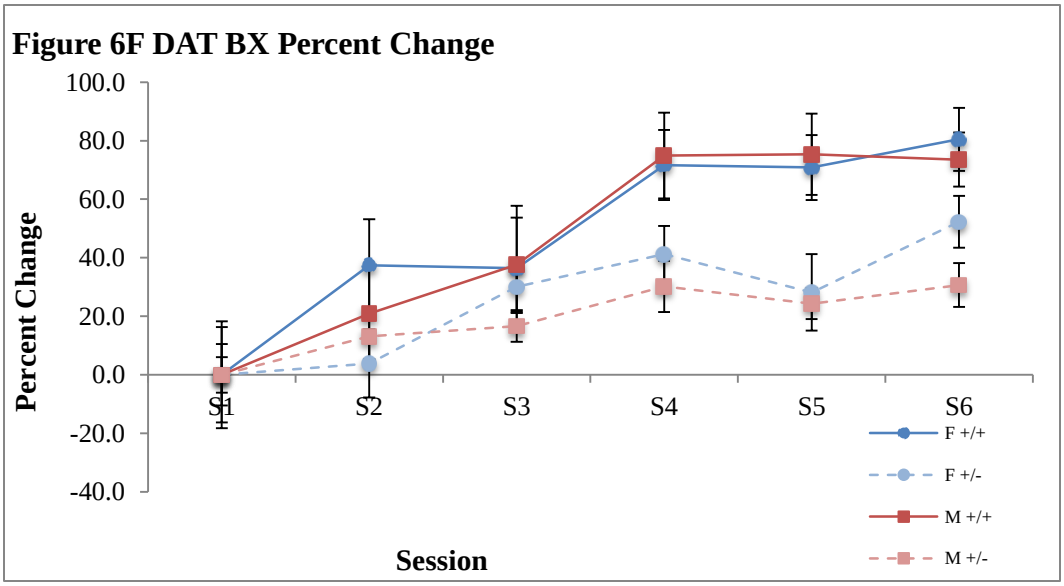


Figure 6E. All groups increased ethanol consumption (g/kg/day), but male +/- increased much less than the other groups. A significant effect of SESSION ($F [5,40] = 21.6$, $p<0.0001$) and significant interaction of SESSION x GENOTYPE ($F [5,40] = 2.8$, $p<0.02$) was observed.

As the main goal of this experiment was to identify differences in escalation of ethanol between genotypes, escalation was evaluated as percent change from the group mean from session one. There was a clear increase for all groups. However, there was greater escalation in WT mice compared with the KO mice, and this observation was supported by the observation of significant effects of GENOTYPE ($F [1,40] = 9.8$, $p<0.004$; Fig 6F) and SESSION ($F [5,40] = 23.8$, $p<0.0001$), and a significant SESSION x GENOTYPE interaction ($F [5,40] = 3.3$, $p<0.0061$) in the ANOVA. It must be noted that this effect of genotype was opposite to the original hypothesis that DAT hKO would increase escalation of ethanol consumption.



Because of the significant effects of SESSION, and significant effects and interactions involving GENOTYPE, analyses were split by SEX and GENOTYPE to further clarify the nature of these changes in each individual group.

Female WT mice showed a significant increase in ethanol solution consumption with SESSION ($F [5,10] = 7.9$, $p<0.0001$). *Post hoc* mean comparisons showed that there was significant increase from session 1 to all other sessions 2-6 ($p<0.02$ for sessions 2-3, $p<0.0001$ for sessions 4-6). There were no significant effects observed for water consumption. Total volume increased significantly with SESSION ($F [5,10] = 4.7$, $p<0.002$). *Post hoc* mean comparisons showed these increases were significant between session 1 and sessions 4-6 ($p<0.002$). Ethanol preference increased from 75% to more than 95%, and a distinct ceiling effect was observed. This increase was significant for SESSION ($F [5,10] = 2.7$, $p<0.03$). *Post hoc* mean comparisons showed significant increases between session 1 and sessions 4-6 ($p<0.009$ for all). Consumption expressed as g/kg/day increased from a starting average of 7.5 g/kg/day to a maximum of 14 g/kg/day, as confirmed by a significant effect of SESSION (F

[5,10] = 7.5, $p<0.0001$), and significant increases from session 1 to all other sessions 2-6 ($p<0.03$ for sessions 2-3, $p<0.0001$ for sessions 4-6) in *post hoc* mean comparisons. Finally, female WT mice showed positive percent increase with session, arriving at a maximum increase of 80% at session 6, as demonstrated by a significant effect of SESSION (F [5,10] = 7.9, $p<0.0001$), and significant increases from session 1 to all subsequent sessions 2-6 ($p<0.02$ for session 2, $p<0.03$ for session 3, and $p<0.0001$ for sessions 4-6) in *post hoc* means comparisons.

Female hKO mice showed similar patterns overall to those previously described. Ethanol solution consumption increased with session, from an initial 3.4 mL to 5.2 mL. This increase was significant, with a significant effect of SESSION (F [5,11] = 5.4, $p<0.0005$), and significant increases observed between session 1 and sessions 2-6 ($p<0.03$). Water consumption decreased with session, from an initial 0.45 mL to 0.27 mL. This was confirmed by a significant effect of SESSION (F [5,11] = 3.7, $p<0.006$), and significant decreases from session 1 to sessions 4-6 ($p<0.01$). Total fluid consumption increased from an initial 3.8 mL to a maximum of 5.3 mL at session 6. A significant effect of SESSION (F [5,11] = 4.4, $p<0.002$) was observed, and the increases were significant from session 1 to sessions 4-6 ($p<0.002$). There were no significant effects involving ethanol preference, which may have been due to ceiling effects. Ethanol consumption, expressed as g/kg/day, increased from an initial 9.6 g to a maximum of 14.6 g at session 6. This increase was significant, with an effect of SESSION (F [5,11] = 4.7, $p<0.002$), and significant *post hoc* mean comparisons between session1 and sessions 3-6 ($p<0.04$). Finally, female hKO mice showed positive percent increases, reaching a maximum increase of 52% increase at session 6. A significant effect of SESSION (F [5,110 = 5.4, $p<0.0004$) was observed, and increases were found significant by *post hoc* individual mean comparisons between session 1 and sessions 3-6 ($p<0.03$).

Similar patterns were seen for male WT mice. Ethanol solution consumption increased from an initial 2.5 mL to 4.5 mL maximum at session 6. This increase was significant, and a significant effect of SESSION ($F [5,8] = 7.2$, $p<0.0001$) was observed. The increases were significant from session 1 to sessions 3-6 ($p<0.04$). No significant effects were observed involving water consumption. Total consumption increased from an initial 3.5 mL to a maximum of 4.8 mL at session 4. A significant effect of SESSION ($F [5,8] = 10.0$, $p<0.0001$) was observed. *Post hoc* comparisons of individual means showed significant increases between session 1 and 3-6 ($p<0.003$). Ethanol preference significantly increased from an initial 71% to a maximum of 94% at session 5. A ceiling effect was also observed for this group. A significant effect of SESSION ($F [5,8] = 2.7$, $p<0.04$) was observed, and significant increases were observed between session 1 and sessions 4-6 ($p<0.01$). Consumption as g/kg/day increased with session from an initial 7.7 g to a maximum of 13.7 g at session 5. This increase was found to be significant, with an effect of SESSION ($F [5,8] = 6.6$, $p<0.0002$), and significant increases observed between session 1 and sessions 3-6 ($p<0.05$). A positive percent increase was observed, with a maximum increase of 75% observed at session 5. These results showed a significant effect of SESSION ($F [5,8] = 7.2$, $p<0.0001$), and the increases were significant between session 1 and sessions 3-6 ($p<0.04$).

Analysis with 1-way ANOVA was consistent with the significant interactions in the initial ANOVA for M hKO mice. Ethanol solution consumption increased with session, from an initial 3.4 mL to a maximum of 4.5 mL at session 6, supported by a significant effect of SESSION ($F [5,11] = 3.1$, $p<0.02$). These increases were significant between session 1 and sessions 4-6 ($p<0.02$). Water consumption significantly decreased from an initial .99 g to .20 grams by session 5-6, supported by an effect of SESSION ($F [5,11] = 4.8$, $p<0.001$). The

decrease in water consumption was significant between session 1 and sessions 2-6 ($p<0.01$). There was no significant change in total fluid consumption. Ethanol preference increased from an initial 79% to a maximum of 95%+ at sessions 5-6. Previously described ceiling effects were seen for male hKO as well, and ethanol preference was significantly affected by SESSION ($F [5,11] = 4.2$, $p<0.003$). These increases were significant between session 1 and sessions 2-6 ($p<0.03$). Consumption as g/kg/day also increased, from an initial 7.9 g to a maximum of 10.3 g at session 6. There was a significant effect of SESSION ($F [5,11] = 2.9$, $p<0.02$), and significant increases were observed between session 1 and sessions 4-6 ($p<0.02$). Male hKO mice showed positive percent increase, with a maximum increase of 31% observed for session 6. There was a significant effect of SESSION ($F [5,11] = 3.1$, $p<0.02$). *Post hoc* comparison of means showed significant increases between session 1 and sessions 4-6 ($p<0.02$).

Because of the significant effects of GENOTYPE observed in the initial ANOVA, *post hoc* mean comparisons were performed to determine the nature of the genotypic effects. Only measures that showed significant effects of GENOTYPE in the initial ANOVA were analyzed in this manner. WT mice showed significantly more consumption as g/kg for session 5 compared to hKO mice ($p<0.05$). WT mice also showed significantly higher levels of percent change than hKO mice for sessions 4-6 ($p<0.002$).

Experiment 2C: VMAT KO

All groups showed increased ethanol solution consumption, increased ethanol preference, increased consumption as g/kg/day, and positive percent increase. The levels of consumption

and preference were comparable to levels observed in the DAT strain, and as such, the levels observed in VMAT mice were less than the DAT BX strain.

Volume of ethanol solution consumed increased across the experiment in a manner that differed between sexes and genotypes. Male and female WT mice showed a trend towards increased differences across the session where female WT mice drank more than male WT mice, but the effect was not significant ($F [1,44] = 2.7$, $p<0.11$). Male and female hKO mice did not show this trend. ANOVA, including SEX and GENOTYPE as factors, revealed a significant effect of SESSION ($F [5,44] = 24.6$, $p<0.0001$; Fig 7A) and a significant SESSION x GENOTYPE x SEX interaction ($F [5,44] = 2.6$, $p<0.03$).

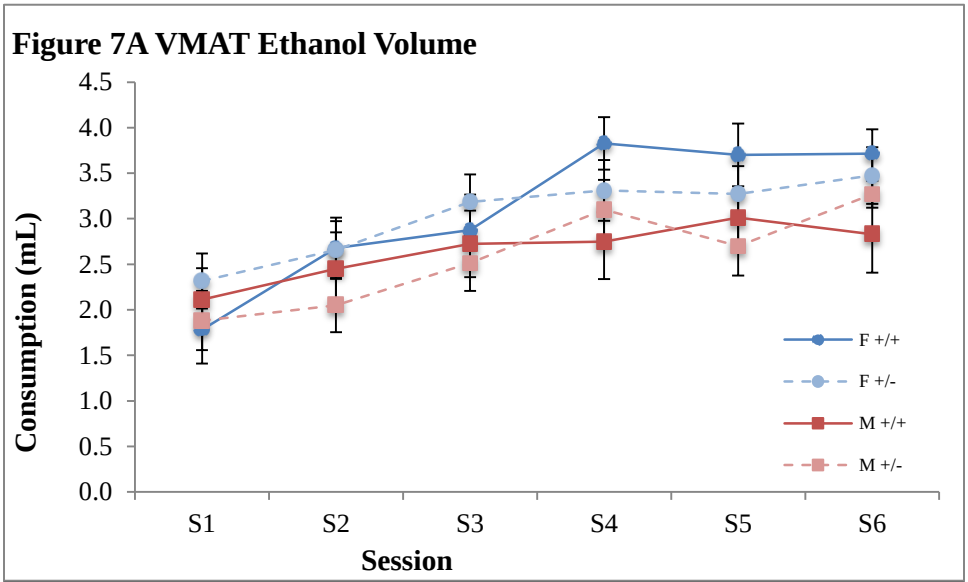


Figure 7A. Ethanol solution consumption increased with session, and a significant effect of SESSION ($F [5,44] = 24.6$, $p<0.0001$) and a significant SESSION x GENOTYPE x SEX interaction ($F [5,44] = 2.6$, $p<0.03$) was observed.

As seen in other experiments, there was a decrease in water consumption across the experiment for all groups, supported by an effect of SESSION ($F [5,44] = 7.1$, $p<0.0001$; Fig 7B).

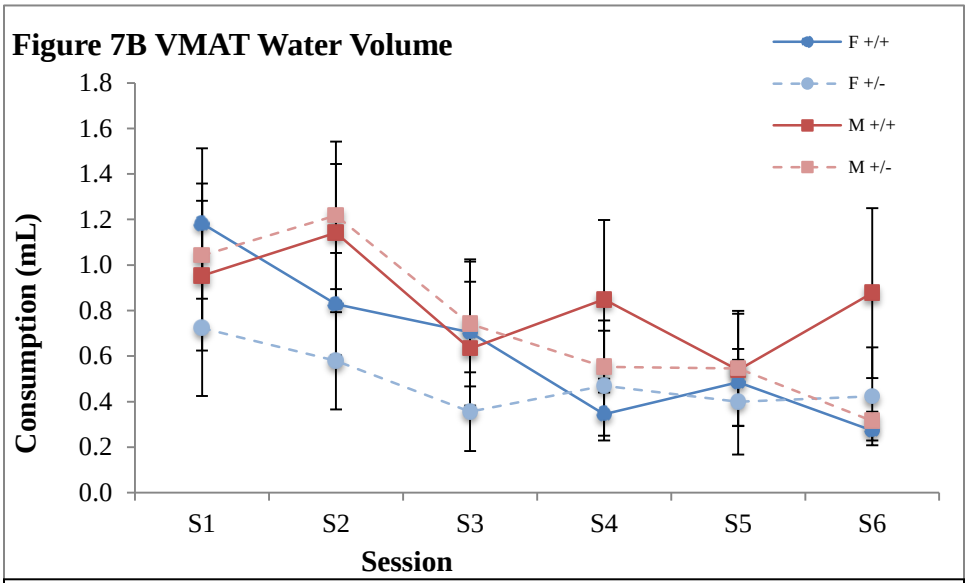


Figure 7B. Water consumption decreased with session, and a significant effect of SESSION ($F [5,44] = 7.1$, $p<0.0001$) was observed.

All groups also increased their total consumption with session, but females increased somewhat more than males. ANOVA showed a significant effect of SESSION ($F [5,44] = 18.8$, $p<0.0001$; Fig 7C) and a significant interaction of SESSION x SEX ($F [5, 44] = 2.5$, $p<0.04$).

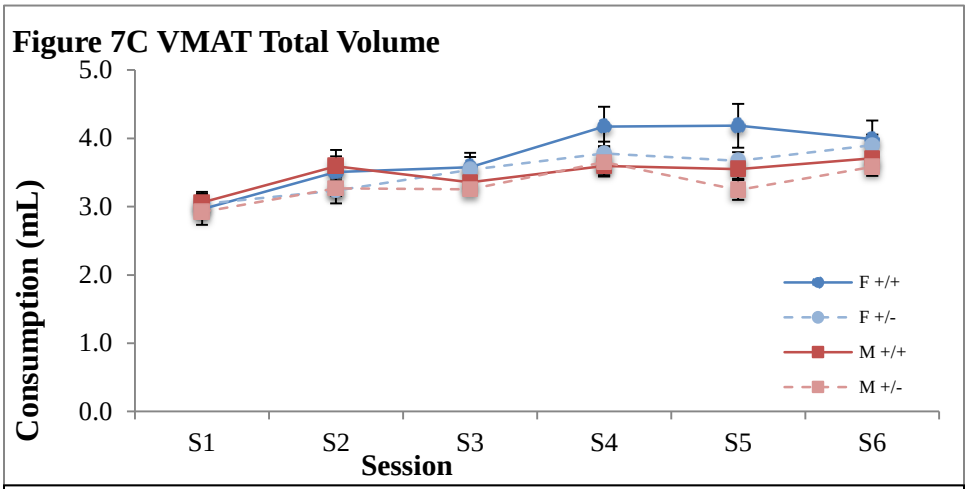


Figure 7C. Total fluid consumption increased with session, and a significant effect of SESSION ($F [5,44] = 18.8$, $p<0.0001$) and an interaction of SESSION x SEX ($F [5, 44] = 2.5$, $p<0.04$) was observed.

Given the above description of increase in ethanol consumption with concurrent decrease in water consumption, it follows that all groups also showed an increase in preference for ethanol across the sessions, and effects of SESSION ($F [5,44] = 9.2$, $p<0.0001$; Fig 7D) and SESSION x GENOTYPE x SEX ($F [5,44] = 2.4$, $p<0.04$).

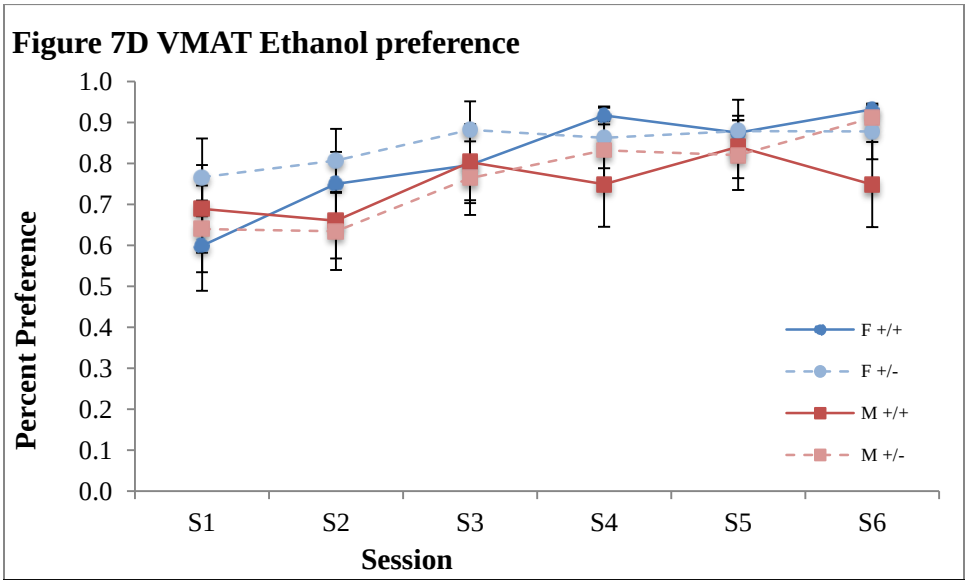


Figure 7D. Preference increased with session, and a ceiling effect was observed. There was a significant effect of SESSION ($F [5,44] = 9.2$, $p<0.0001$) and a significant interaction of SESSION x GENOTYPE x SEX ($F [5,44] = 2.4$, $p<0.039$).

For the final three sessions, female WT mice showed significantly more consumption compared to male WT mice. Female hKO showed higher initial levels of consumption compared to all groups, but in the later sessions, they did not show a great difference from males. When ethanol consumption was calculated as g/kg/day, effects of SESSION ($F [5, 44] = 36.5$, $p<0.0001$; Fig 7E) and SESSION x GENOTYPE * SEX ($F [5,44] =2.8$, $p<0.02$) were observed. All groups increased their ethanol consumption across the sessions.

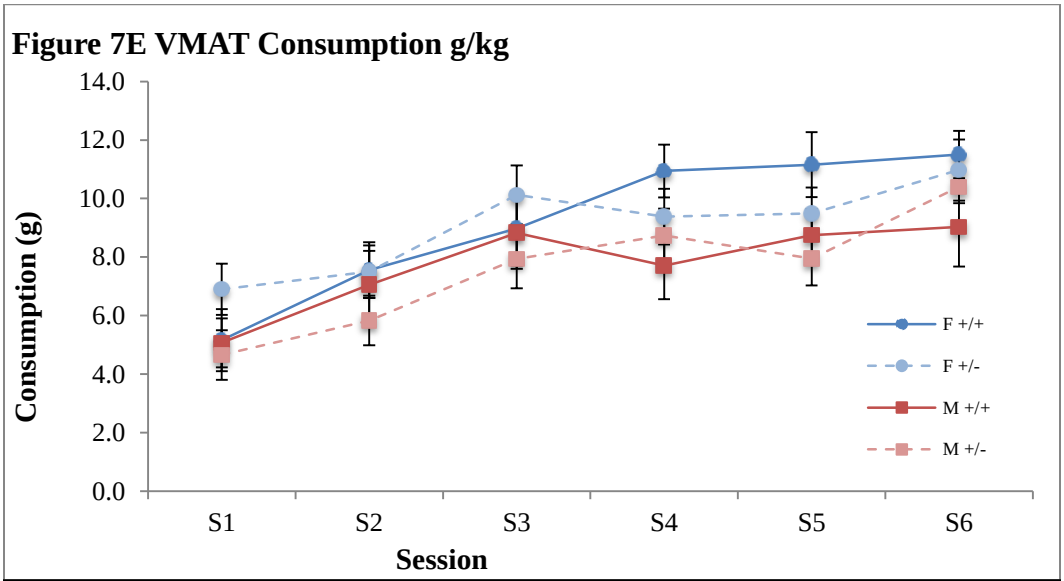


Figure 7E. Consumption as g/kg/day increased as session increased, and a significant effect of SESSION ($F [5,44] = 9.2$, $p<0.0001$) and interaction of SESSION x GENOTYPE x SEX ($F [5,44] = 2.4$, $p<0.04$) were observed.

Positive percent change in ethanol consumption was observed for all VMAT mice. First, an effect of SESSION ($F [5,44] = 24.6$, $p<0.0001$; 7F) as all groups increased over the sessions. There were also significant interactions between GENOTYPE x SEX ($F [1, 44] = 4.1$, $p<0.048$) and SESSION x GENOTYPE x SEX ($F [5,44] = 4.0$, $p<0.0016$), where female WT mice increased significantly more than all over groups, however, for sessions 4 and 6 male hKO mice showed higher percent change compared with male WT and female hKO.

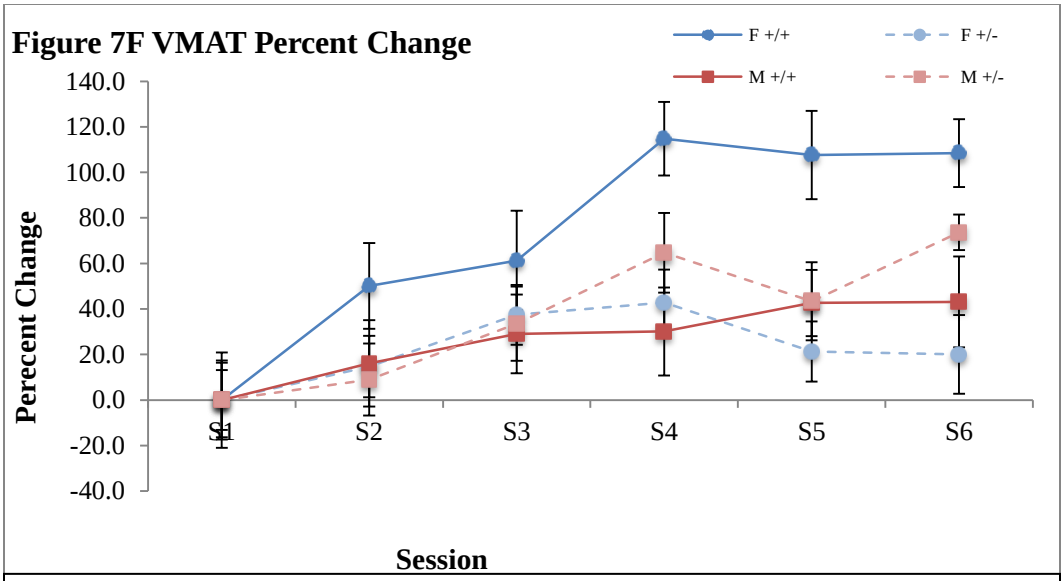


Figure 7F. All groups increased consumption with session. There was a significant effect of SESSION (F [5,44] = 24.6, p<0.0001) and significant interactions between GENOTYPE x SEX (F [1, 44] = 4.1, p<0.048) and SESSION x GENOTYPE x SEX (F [5,44] = 4.0, p<0.0016).

As significant effects and interactions involving SEX were identified in the initial ANOVA, analyses were further split by sex. When ethanol solution consumption was analyzed, a significant effect of SESSION (F [5,22] = 17.6, p<0.0001) was observed, consistent with previous observations that ethanol solution consumption increased with session. As initial analysis did not find an effect or interaction involving SEX related to water consumption, results are not presented from this ANOVA for that measure. Total fluid consumption increased with session, and there was a significant effect of SESSION (F [5,22] = 13.0, p<0.0001) for this measure. Ethanol preference increased with session, and a maximum, for all groups, was observed at 87% by session 4 and maintained for subsequent sessions, suggesting potential ceiling effects for this measure. The increase in preference was significant, supported by a significant effect of SESSION (F [5,22] = 6.5, p<0.0001). Consumption expressed as g/kg/day also increased with session. Female WT mice showed slightly lower initial levels of consumption, but then surpassed female hKO consumption for sessions 4 and 5. These

observations are supported by a significant effect of SESSION ($F [5,22] = 20.4$, $p<0.0001$) and a significant SESSION x GENOTYPE interaction ($F [5,22] = 2.4$, $p<0.04$). The most marked genotypic differences were observed in the percent change measure, where female WT mice escalated to a maximum of 114% increase by session 4, compared with female hKO maximum increase of 50% at session 6. Part of this effect could be due to the initial higher consumption in female hKO mice, as they would have to increase their consumption more to achieve similar percent change. Nonetheless, significant effects of GENOTYPE ($F [5,22] = 5.3$, $p<0.04$) and SESSION ($F [5,22] = 17.1$, $p<0.0001$), along with a significant SESSION x GENOTYPE ($F [5,22] = 3.3$, $p<0.009$) interaction were observed.

Further analysis was warranted by the observation of significant effects of SESSION, and interactions with GENOTYPE, in the original ANOVA. Analyses were further split by sex and genotype in *post hoc* analyses. Analysis of data from female WT mice was consistent with results identified in previous analyses. Ethanol solution consumption increased with session from an initial 1.8 mL to a maximum of 3.8 mL at session 4 (consumption was 3.7 mL for sessions 5-6), and there was a significant effect of SESSION ($F [5,11] = 11.1$, $p<0.0001$) observed. *Post hoc* individual means comparisons showed these increases to be significant between session 1 and sessions 2-6 ($p<0.02$). Water consumption decreased as ethanol solution consumption increased, from an initial 1.2 mL to a minimum of 0.27 mL at session 6. This decrease was significant, supported by a significant effect of SESSION ($F [5,11] = 3.7$, $p<0.006$) and significant *post hoc* mean comparison decreases between session 1 and sessions 4-6 ($p<0.008$). Total consumption increased from an initial 3.0 mL to a maximum of 4.2 mL at session 5. A significant effect of SESSION ($F [5,11] = 8.3$, $p<0.0001$) was observed, and significant increases were observed between session 1 and sessions 2-6 ($p<0.02$). Given the

above description, it follows that a significant increase in ethanol preference was observed, from an initial preference of 60% to a maximum of 93% at session 6. These levels of preference are similar to levels observed for DAT and DAT BX strains, and are suggestive of a ceiling effect. There was a significant effect of SESSION ($F [5,11] = 5.1$, $p<0.0007$) on preference, and the increases were significant between session 1 and sessions 3-6 ($p<0.02$). Consumption as g/kg/day showed similar increases, where initial consumption was 5.1 g/kg and a maximum of 11.5 g/kg consumption was observed at session 6. There was a significant effect of SESSION ($F [5,11] = 12.6$, $p<0.0001$), and significant increases in g/kg consumption were observed between session 1 and sessions 2-6 ($p<0.02$). Lastly, female WT mice showed positive percent increase, achieving a maximum of 114% increase at session 4 (comparable levels of increase were observed for sessions 5-6—107% and 108% respectively). There was a significant effect of SESSION ($F [5,11] = 11.1$, $p<0.0001$), and significant increases in percent change were observed between session 1 and sessions 2-6 ($p<0.02$).

Analysis of data from female hKO mice was also consistent with results identified in previous analyses. Ethanol solution consumption increased with session from an initial 2.3 mL to a maximum of 3.4 mL at session 6 (the initial level was higher than female WT mice, but the maximum was less than female WT mice), and there was a significant effect of SESSION ($F [5,11] = 7.0$, $p<0.0001$) observed. *Post hoc* individual mean comparisons showed these increases to be significant between session 1 and sessions 3-6 ($p<0.0007$). Water consumption showed no significant change. Total consumption increased from an initial 3.0 mL to a maximum of 3.9 mL at session 6, which are similar levels to female WT mice. A significant effect of SESSION ($F [5,11] = 5.2$, $p<0.0006$) was observed, and significant increases were observed between session 1 and sessions 2 and 4-6 ($p<0.008$). No significant effects were

observed in analysis of preference. Consumption as g/kg/day increased, where initial consumption was 6.9 g/kg and a maximum of 10.9 g/kg consumption was observed at session 6. There was a significant effect of SESSION ($F [5,11] = 9.3$, $p<0.0001$), and significant increases in g/kg consumption were observed between session 1 and sessions 3-6 ($p<0.002$). Lastly, female hKO mice showed positive percent increase, achieving a maximum of 50% increase at session 6, which was less than half of the 114% maximum increase observed for female WT mice. There was a significant effect of SESSION ($F [5,11] = 7.0$, $p<0.0001$), and significant increases in percent change were observed between session 1 and sessions 3-6 ($p<0.0007$).

Data from male WT was similarly analyzed. Ethanol solution consumption increased with session from an initial 2.1 mL to a maximum of 3.0 mL at session 5, and there was a significant effect of SESSION ($F [5,11] = 2.5$, $p<0.04$) observed. *Post hoc* mean comparisons showed these increases to be significant between session 1 and sessions 3-6 ($p<0.04$). There was no significant effect involving water consumption. Total consumption increased from an initial 3.0 mL to a maximum of 3.7 mL at session 6. A significant effect of SESSION ($F [5,11] = 3.5$, $p<0.008$) was observed, and significant increases were observed between session 1 and sessions 2 and 4-6 ($p<0.004$). No significant effects involving ethanol preference were observed. Consumption as g/kg/day increased, where initial consumption was 5.1 g/kg and a maximum of 9.0 g/kg consumption was observed at session 6. There was a significant effect of SESSION ($F [5,11] = 6.7$, $p<0.0001$), and significant increases in g/kg consumption were observed between session 1 and sessions 2-6 ($p<0.02$). Lastly, male WT mice showed positive percent increase, achieving a maximum of 43% increase at session 5. There was a significant effect of SESSION ($F [5,11] = 2.5$, $p<0.04$), and significant increases in percent change were observed between session 1 and sessions 3-6 ($p<0.04$).

The final analysis of male hKO mice was consistent with results identified in previous analyses. Ethanol solution consumption increased with session from an initial 1.9 mL to a maximum of 3.2 mL at session 6, which are similar levels seen in male WT mice. A significant effect of SESSION ($F [5,11] = 6.9$, $p<0.0001$) was identified, and *post hoc* mean comparisons showed these increases to be significant between session 1 and sessions 3-6 ($p<0.04$). Water consumption decreased as ethanol solution consumption increased, from an initial 1.0 mL to a minimum of 0.32 mL at session 6, which is similar to the significant decrease noted in female VMAT WT mice. This decrease was significant, supported by a significant effect of SESSION ($F [5,11] = 3.1$, $p<0.02$) and a significant *post hoc* decrease between session 1 and session 6 ($p<0.01$). Total consumption increased from an initial 2.9 mL to a maximum of 3.7 mL at session 4 (3.6 mL at session 6). A significant effect of SESSION ($F [5,11] = 4.1$, $p<0.003$) was observed, and significant increases were observed between session 1 and sessions 4 and 6 ($p<0.0003$). Given the above description, it follows that a significant increase in ethanol preference was observed, from an initial preference of 64% to a maximum of 91% at session 6, which are similar levels as seen in female VMAT WT mice. These levels of preference are similar to levels observed for DAT and DAT BX strains, and are suggestive of a ceiling effect. There was a significant effect of SESSION ($F [5,11] = 3.3$, $p<0.01$) on preference, and the increases were significant between session 1 and sessions 4-6 ($p<0.04$). Consumption as g/kg/day showed similar increases, where initial consumption was 4.6 g/kg and a maximum of 10.2 g/kg consumption was observed at session 6. There was a significant effect of SESSION ($F [5,11] = 11.1$, $p<0.0001$), and significant increases in g/kg consumption were observed between session 1 and sessions 3-6 ($p<0.0005$). Lastly, male hKO mice showed positive percent increase, achieving a maximum of 74% increase at session 6. There was a significant effect of SESSION

($F [5,11] = 6.8$, $p<0.0001$), and significant increases in percent change were observed between session 1 and sessions 3-6 ($p<0.04$ for session 3, $p<0.0003$ for session 4, $p<0.009$ for sessions 5, and $p<0.0001$ for session 6).

Due to significant effects of GENOTYPE seen in the initial ANOVA, further *post hoc* mean comparisons were performed to identify the nature of the genotypic differences. While genotypic differences were observed in the initial ANOVA for ethanol consumption, ethanol preference, consumption as g/kg, and percent change, only the latter showed a significant difference in *post hoc* mean comparisons—WT mice showed higher percent change than hKO mice for session 5 ($p<0.05$).

Experiment 2D: VMAT BX

Overall VMAT BX ethanol solution consumption was similar in magnitude to DAT BX mice, and higher that VMAT KO mice. Total levels of fluid consumption were similar in VMAT and VMAT BX mice, although female VMAT BX mice showed higher total fluid consumption (about 1 mL per day higher) than other mice. VMAT BX mice showed high initial levels of ethanol preference, particularly female WT mice, however, there was not as clear of an increasing trend as observed in other strains. VMAT BX mice showed similar levels of initial ethanol consumption (g/kg/day) to DAT BX mice, which also meant that VMAT BX mice showed higher initial levels of consumption than VMAT KO mice. Final levels of consumption were higher in VMAT BX mice than VMAT KO mice, and this was particularly apparent in female VMAT BX WT mice. In both VMAT KO and VMAT BX mice, female WT mice showed markedly higher levels of consumption expressed as percent increase compared with all

other groups for this strain, and in fact VMAT female WT mice showed a higher maximum percent increase than VMAT BX female WT mice (114% compared to 74%). As with all other strains for this escalation paradigm, all groups showed increased consumption of ethanol across sessions. Female WT mice showed distinctly higher consumption of the ethanol solution after session 1, with significance at session 4. Males consumed similar amounts of ethanol, regardless of genotype. Analysis revealed effects of SESSION ($F [5,44] = 19.4$, $p<0.0001$; Fig 8A), SESSION x GENOTYPE ($F [5,44] = 5.0$, $p<0.0002$; Fig 8A), and SESSION x SEX ($F [5,44] = 3.2$, $p<0.008$; Fig 8A).

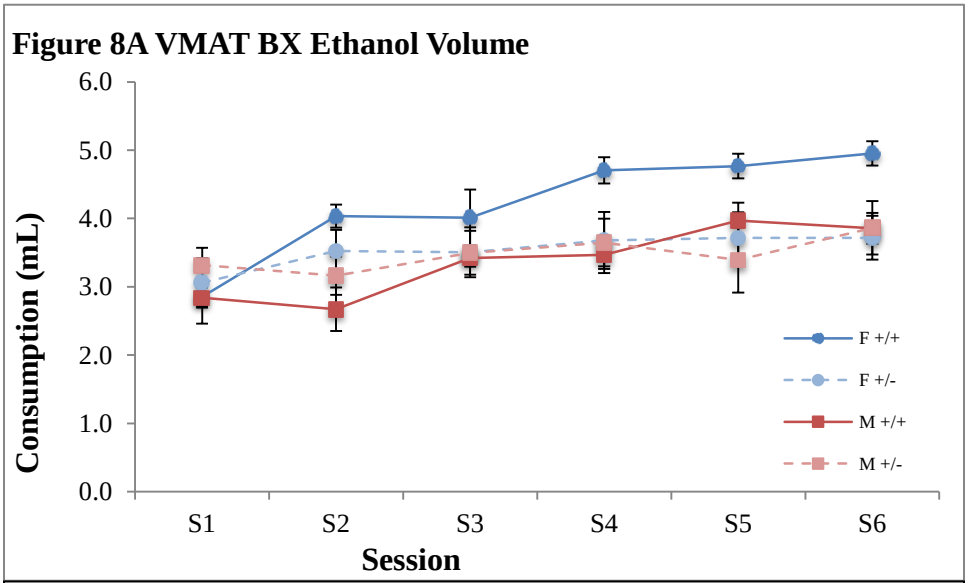


Figure 8A. All groups increased their ethanol consumption, but female WT showed higher increases than any other group. A significant effect of SESSION ($F [5,44] = 19.4$, $p<0.0001$), and significant interactions of SESSION x GENOTYPE ($F [5,44] = 5.0$, $p<0.0002$), and SESSION x SEX ($F [5,44] = 3.2$, $p<0.008$) were observed.

Female WT mice showed minimal water consumption, while male WT mice showed higher, more variable water consumption that showed a decreasing trend. Both male and female hKO mice showed similar levels of water consumption, which marginally decreased with session, but remained highly variable. These observations are supported by a significant effect

of SESSION (F [5,44] = 3.3, p<0.007; Fig 8B), and a significant interaction of SESSION x GENOTYPE x SEX (F [5,44] = 2.8, p<0.02).

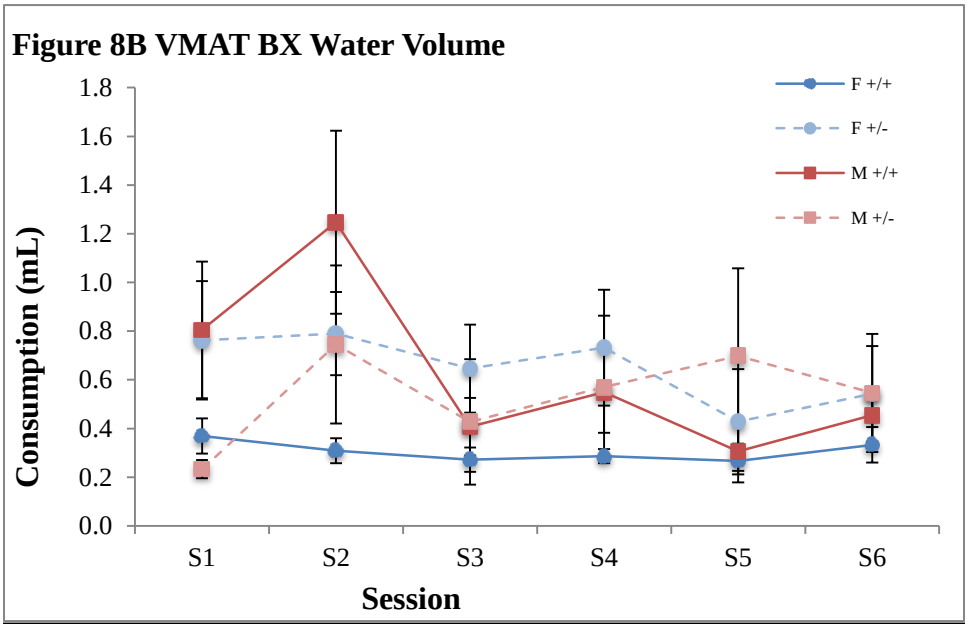


Figure 8B. Water consumption was highly variable for most groups, although there was a general trend towards decreased water consumption. F WT mice showed minimal water consumption. A significant effect of SESSION (F [5,44] = 3.3, p<0.007), and a significant interaction of SESSION x GENOTYPE x SEX (F [5,44] = 2.8, p<0.02) was observed.

Total consumption increased for all groups, but again this effect was more prominent in female WT mice. All other groups increased consumption at similar levels. A significant effect of SESSION (F [5,44] = 20.8, p<0.0001; Fig 8C) and significant interactions of SESSION x GENOTYPE (F [5,44] = 3.5, p<0.004) and SESSION x GENOTYPE x SEX (F [5,44] = 4.2, p<0.001) were observed.

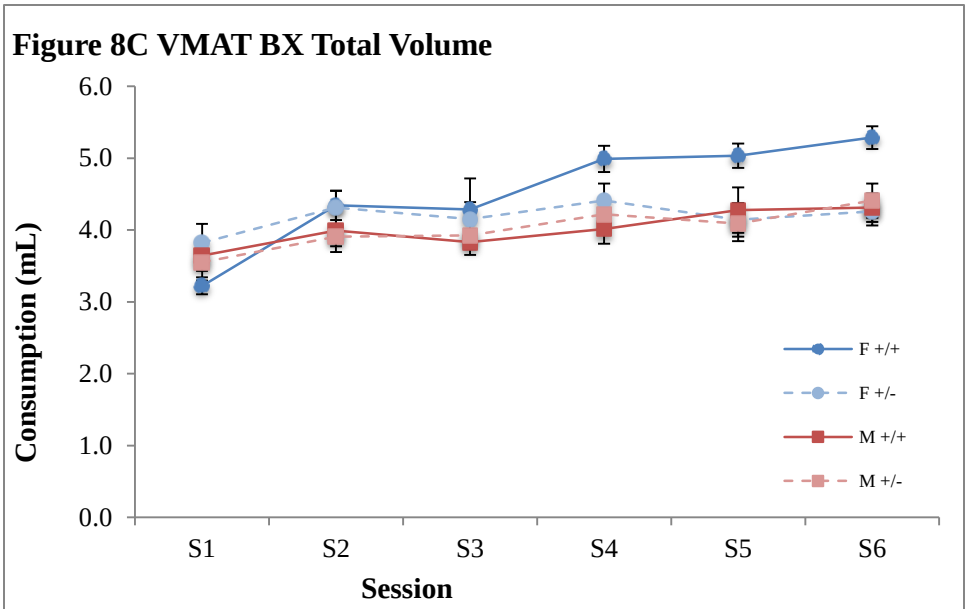


Figure 8C. All mice increased their total fluid consumption, but this increase was higher in F WT mice. A significant effect of SESSION ($F [5,44] = 20.8$, $p<0.0001$) and significant interactions of SESSION x GENOTYPE ($F [5,44] = 3.5$, $p<0.004$) and SESSION x GENOTYPE x SEX ($F [5,44] = 4.2$, $p<0.001$) were observed.

Ethanol preference was somewhat varied, in part because VMAT BX mice showed the highest levels of preference for any strain. There was minimal room for the mice to escalate preference, particularly in the case of female WT mice. Nonetheless, a significant effect of SESSION ($F [5,44] = 2.7$, $p<0.02$; Fig 8D) and a significant interaction of SESSION x GENOTYPE x SEX ($F [5,44] = 2.8$, $p<0.02$), describing the differences between sessions and the difference of female WT mice from the rest of the groups.

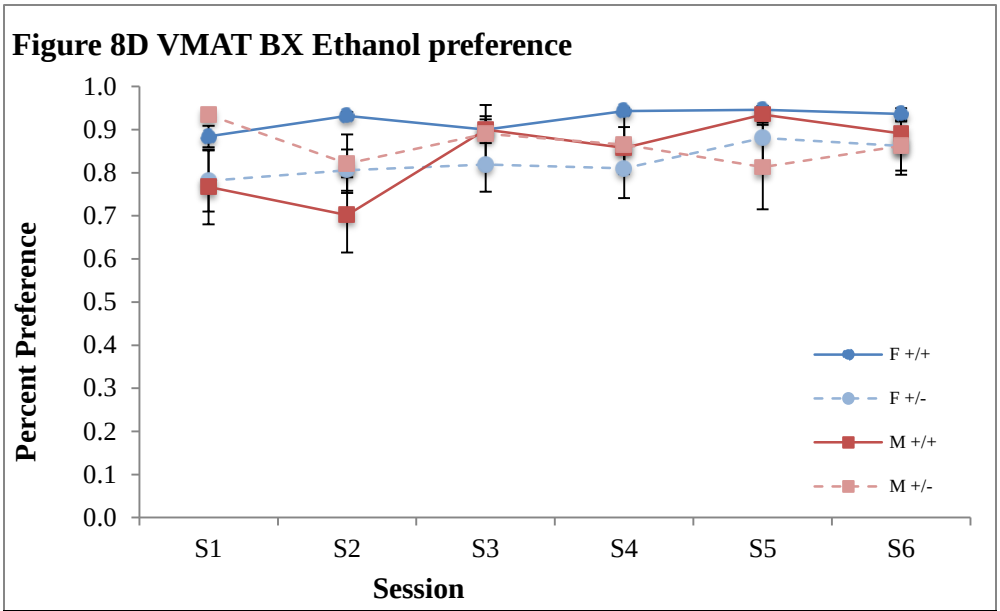


Figure 8D. Ethanol preference was variable, but there was a trend towards increased preference, and F WT mice showed elevated preference. A significant effect of SESSION ($F [5,44] = 2.7$, $p<0.02$) and a significant interaction of SESSION x GENOTYPE x SEX ($F [5,44] = 2.8$, $p<0.02$) was observed.

When consumption was analyzed as g/kg/day, a distinct increase in ethanol consumption was observed for all groups. Again, larger increases were observed for female WT consumption, and males, regardless of genotype, showed similar levels of consumption to female hKO mice. It follows that significant effects of SEX ($F [1,44] = 4.3$, $p<0.05$) and SESSION ($F [5,44] = 50.1$, $p<0.0001$), along with a significant interaction of SESSION and GENOTYPE ($F [5,44] = 3.7$, $p<0.003$) was observed.

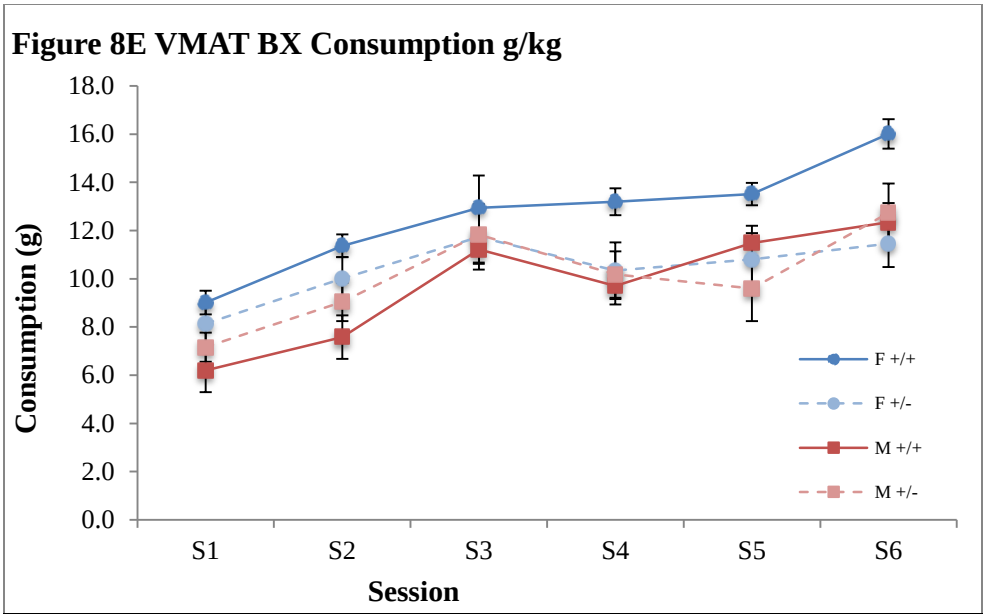


Figure 8E. Consumption as g/kg increased for all mice, and this increase higher in F WT mice. A significant effect of SESSION (F [5,44] = 50.1, p<0.0001), along with a significant interaction of SESSION x GENOTYPE (F [5,44] = 3.7, p<0.003) was observed.

All VMAT BX mice showed positive percent increase with session. WT mice showed higher levels of increase than hKO mice, and females generally consumed more than males of the same genotype. As observed with other measures of ethanol consumption in this experiment, female WT mice escalated to a higher extent than any of the other mice. Significant effects of GENOTYPE (F [1,44] = 7.5, p<0.009; Fig 8F), SEX (F [1,44] = 5.6, p<0.03), and SESSION (F [5,44] = 20.3, p<0.0001), along with significant interactions of SESSION and GENOTYPE (F [5,44] = 6.1, p<0.0001) and SESSION and SEX (F [5,44] = 3.4, p<0.006).

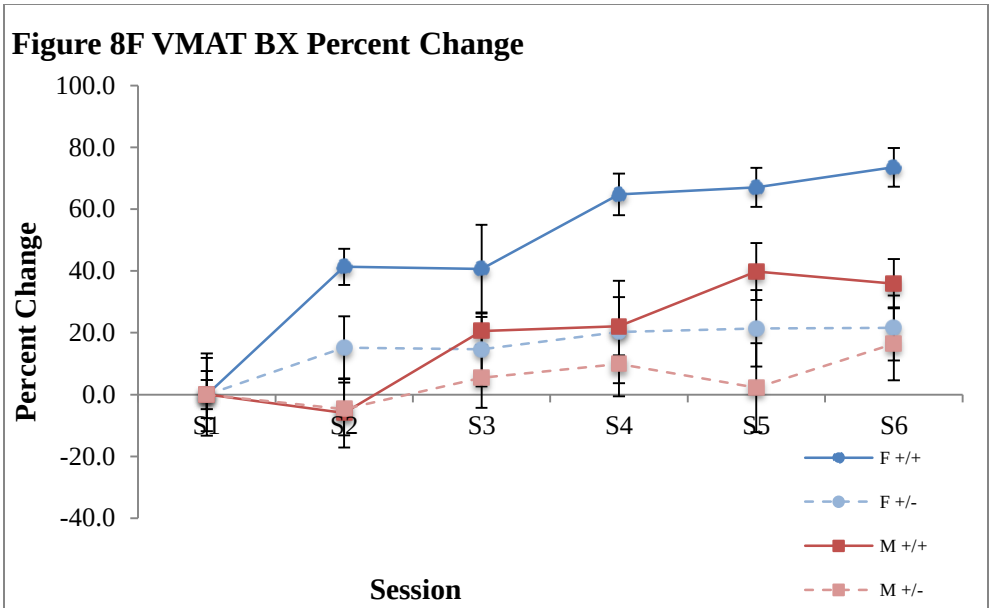


Figure 8F. Consistent with trends observed for other measures in this strain, all mice showed positive increase in consumption, but F WT mice showed the largest increases. Significant effects of GENOTYPE ($F [1,44] = 7.5$, $p<0.009$), SEX ($F [1,44] = 5.6$, $p<0.03$), and SESSION ($F [5,44] = 20.3$, $p<0.0001$), along with significant interactions of SESSION and GENOTYPE ($F [5,44] = 6.1$, $p<0.0001$) and SESSION and SEX ($F [5,44] = 3.4$, $p<0.006$) were observed.

Due to the significant effects and interactions involving SEX in the initial ANOVA, data were split by SEX and then analyzed. Ethanol consumption increased for all female mice, but female WT mice increased to higher levels than hKO females. There was a significant effect of SESSION ($F [5,22] = 15.9$, $p<0.0001$) and a significant interaction of SESSION and GENOTYPE ($F [5,44] = 4.2$, $p<0.002$). There were no significant effects or interactions involving water consumption. Female hKO mice started at higher levels of total consumption for session 1 (3.2 mL vs. 3.8 mL), but female WT mice consumed equivalent levels for sessions 2-3, and increased their consumption to higher levels than the F hKO for sessions 4-6. Analysis of this data showed a significant effect of SESSION ($F [5,22] = 17.1$, $p<0.0001$) and a significant interaction of SESSION x GENOTYPE ($F [5,44] = 7.4$, $p<0.0001$). No significant effects or interactions were observed involving ethanol preference. When consumption as g/kg was evaluated, both female WT and female hKO mice increased the magnitude of their ethanol

consumption, but female WT mice increased to a greater extent. This genotypic difference was more apparent at sessions 4-6. Significant effects of GENOTYPE ($F [1,22] = 4.3$, $p<0.05$) and SESSION ($F [5,22] = 19.9$, $p<0.0001$), along with a significant interaction of SESSION x GENOTYPE ($F [5,44] = 3.1$, $p<0.02$) were observed. All females showed positive levels of percent increase across sessions, but F WT mice showed over 3 times the escalation of female hKO mice for sessions 4-6. It is no surprise that significant effects of GENOTYPE ($F [1,22] = 7.4$, $p<0.02$) and SESSION ($F [5,22] = 16.3$, $p<0.0001$) along with a significant interaction of SESSION x GENOTYPE ($F [5,22] = 4.8$, $p<0.0006$) were observed.

Males showed similar levels of ethanol consumption, and increased their consumption across sessions. A significant effect of SESSION ($F [5,22] = 7.1$, $p<0.0001$) was observed. While water consumption was more variable than seen in other strains, there was a trend in decreasing water consumption as session increased. For the first two sessions, male WT mice showed higher levels of water consumption, but reduced their water consumption to levels similar to male hKO mice for subsequent sessions. A significant effect of SESSION ($F [5,22] = 3.0$, $p<0.02$) and a significant interaction of SESSION x GENOTYPE ($F [5,22] = 2.4$, $p<0.04$) was observed. Males showed similar levels of total fluid consumption, regardless of genotype, and increased total fluid consumption with session. Thus, a significant effect of SESSION ($F [5,22] = 5.7$, $p<0.0001$) on total fluid consumption was observed. Male hKO mice showed elevated initial ethanol preference, but preference levels comparable between male hKO and male WT mice by session 3. As previously seen in other strains, a ceiling effect was observed. Analysis revealed a significant effect of SESSION ($F [5,22] = 2.6$, $p<0.03$) and a significant SESSION x GENOTYPE interaction ($F [5,22] = 3.2$, $p<0.01$). When consumption as g/kg/day was evaluated, there was a trend towards increased consumption in male hKO mice compared

with M WT mice for sessions 1 and 2, and then consumption levels became similar for all males. As seen in female VMAT BX and other strains, consumption as g/kg/day increased across sessions. A significant effect of SESSION ($F [5,22] = 31.5$, $p<0.0001$) and a significant SESSION x GENOTYPE interaction ($F [5,22] = 2.3$, $p<0.05$) was observed. All males showed a positive percent change by session 3, although male WT mice showed higher levels of increase compared with male hKO mice. Thus, a significant effect of SESSION ($F [5,22] = 7.5$, $p<0.0001$) and a significant SESSION x GENOTYPE interaction ($F [5,22] = 2.6$, $p<0.03$) was observed.

Due to the significant effects and interactions involving SESSION in the initial ANOVA, subsequent ANOVAs were performed, split by SEX and GENOTYPE. Female WT mice showed increased ethanol consumption, from an initial 2.8 mL to a maximum of 4.9 mL at session 6. A significant effect of SESSION ($F [5,11] = 16.1$, $p<0.0001$) was observed, and *post hoc* mean comparisons showed significant increases from session 1 to sessions 2-6 ($p<0.0001$ for all). No significant effect involving water consumption was observed. Total volume increased from an initial 3.2 mL to a maximum of 5.3 mL at session 6. ANOVA showed a significant effect of SESSION ($F [5,11] = 6.8$, $p<0.0001$), and *post hoc* mean comparisons showed significant increases from session 1 to sessions 2-6 ($p<0.0002$ for session 2, $p<0.0004$ for session 3, and $p<0.0001$ for sessions 4-6). Due to initially high (88%) levels of preference, the increase to a maximum of 95% preference at session 5 was not significant ($p>0.05$), likely caused by a ceiling effect. Consumption as g/kg increased from an initial 9.0 g to a maximum level of 16.0 g at session 6. A significant effect of SESSION ($F [5,11] = 14.1$, $p<0.0001$) was observed, and *post hoc* mean comparisons showed significant increases from session 1 to sessions 2-6 ($p<0.01$ for session 2, $p<0.0001$ for all subsequent sessions). Female WT mice showed a maximum

percent change at session 6, reaching 74% increase. Percent increase was significantly affected by SESSION ($F [5,11] = 16.1$, $p<0.0001$), and *post hoc* mean comparisons showed significant increases from session 1 to sessions 2-6 ($p<0.0001$ for all).

There were no significant effects involving ethanol or water consumption in female hKO mice. Total volume increased with session, from an initial level of 3.2 mL to a maximum of 5.3 mL at session 6. A significant effect of SESSION ($F [5,11] = 3.3$, $p<0.01$) was observed, and *post hoc* means comparisons showed significant increases from session 1 to sessions 2-6 ($p<0.0002$ for session 2, $p<0.0004$ for session 3, $p<0.0001$ for sessions 4-6). No significant effect involving ethanol preference was observed. Consumption as g/kg increased with session, from an initial level of 9.0 g to a maximum of 16.0 g reached at session 6. There was a significant effect of SESSION ($F [5,22] = 14.1$, $p<0.0001$), and *post hoc* mean comparisons showed significant increases between session 1 and sessions 2-6 ($p<0.008$ for session 2, $p<0.0001$ for session 3, $p<0.003$ for session 4, $p<0.0003$ for session 5, and $p<0.0001$ for session 6).

Male WT mice showed increased ethanol consumption, from an initial 2.8 mL to a maximum of 4.0 g at session 5. A significant effect of SESSION ($F [5,11] = 7.4$, $p<0.0001$) was observed, and *post hoc* mean comparisons showed significant increases from session 1 to sessions 3-6 ($p<0.04$ for sessions 3, $p<0.03$ for session 4, $p<0.0001$ for session 5, and $p<0.0005$ for session 6). Water consumption decreased from a maximum at session 2 from 1.2 mL to a minimum of 0.3 mL at session 5. A significant effect of SESSION ($F [5,11] = 3.8$, $p<0.005$) was observed, but *post hoc* individual mean comparisons showed no significant changes between sessions. There were no significant effects involving total fluid consumption. Ethanol preference increased with session, from a minimum of 70% at session 2 to a maximum of 94% at

session 5. A significant effect of SESSION ($F [5,11] = 4.4$, $p<0.002$), and *post hoc* means comparisons showed significant increases between session 1 and sessions 3 and 5-6 ($p<0.04$ for session 3, $p<0.008$ for session 5, and $p<0.05$ for session 6). Consumption as g/kg increased from an initial 6.2 g to a maximum level of 12.3 g at session 6. A significant effect of SESSION ($F [5,11] = 20.1$, $p<0.0001$) was observed, and *post hoc* mean comparisons showed significant increases from session 1 to sessions 3-6 ($p<0.0001$ for all). Male WT mice showed a maximum percent change at session 6, reaching 40% increase. Percent increase was significantly affected by SESSION ($F [5,11] = 7.4$, $p<0.0001$), and *post hoc* mean comparisons showed significant increases from session 1 to sessions 3-6 ($p<0.04$ for session 3, $p<0.03$ for session 4, $p<0.0002$ for session 5, and $p<0.0005$ for session 6).

No significant effects involving ethanol or water consumption were observed in male hKO mice. Total volume increased with session, from an initial 3.5 mL to a maximum of 4.4 mL at session 6. A significant effect of SESSION ($F [5,11] = 5.0$, $p<0.0008$) was observed, with *post hoc* mean comparisons showing significant increase between session 1 and sessions 3-6 ($p<0.05$ for session 3, $p<0.0008$ for session 4, $p<0.006$ for session 5, and $p<0.0001$ for session 6). No significant effect involving ethanol preference was observed. Consumption as g/kg increased with session, from an initial 7.1 mL to a maximum of 12.7 mL achieved at session 6. A significant effect of SESSION ($F [5,11] = 13.7$, $p<0.0001$) was observed, and *post hoc* mean comparisons showed significant increases between session 1 and sessions 2-6 ($p<0.02$ for session 2, $p<0.0001$ for session 3, $p<0.0003$ for session 4, $p<0.003$ for session 5, and $p<0.0001$ for session 6). No significant effects were observed involving percent change.

Given the significant effects of GENOTYPE observed in the initial ANOVA, *post hoc* mean comparisons were performed for all measures showing these significant effects. Through

these analyses, significant increases in ethanol consumption for WT mice were observed for sessions 5 and 6 ($p<0.05$). WT also showed elevated total fluid consumption compared with hKO mice for sessions 5 and 6 ($p<0.04$). Consumption as g/kg showed similar effects of elevated consumption in WT mice, which was found significant for sessions 5 and 6 ($p<0.04$). Finally, WT mice showed higher levels of percent increase, significant for sessions 4-6 ($p<0.01$).

DISCUSSION

Summary

In the baseline consumption experiments, all strains showed an effect of concentration on ethanol solution consumption, where solution consumption increased for low to moderate (2-8%) and decreased from moderate to high (16-32%) concentrations. However, because solutions were presented in increasing concentrations, ethanol consumption as g/kg/day increased with concentration. In DAT, DAT BX, and VMAT BX, females consumed more ethanol solution, had higher ethanol preference, and showed higher levels of g/kg ethanol consumption compared with males. The only strain to show any effect of genotype was the VMAT BX strain, where WT mice consumed more ethanol than hKO mice—this effect was greater in males than females. The congenic strain of each KO line showed elevated levels of consumption and preference, consistent with the original hypothesis. When sex effects were observed, females consumed more ethanol than males, which is also consistent with the original hypotheses. However, the genotypic difference observed in VMAT BX mice, where WT mice consumed more ethanol than hKO mice, which opposes the original hypothesis in relation to the effects of VMAT2 KO.

In the second experiment, all strains voluntarily increased their ethanol consumption when presented with access for 24 hours, 2 times per week. For all strains, there was a tendency

towards maximizing ethanol solution preference (i.e. ceiling effects) during the escalation experiments. In the mixed DAT strain, females had markedly higher levels of ethanol consumption (particularly as g/kg/day), and somewhat higher levels of ethanol preference. However, when rate of escalation was calculated as percent change, males showed higher levels of increased escalation when compared with females. In DAT BX mice, again all mice escalated ethanol consumption, but male hKO mice showed lower levels of consumption. In DAT BX mice, there were no significant effects of SEX involved in percent change, but WT mice escalated to a greater extent than hKO mice. In the mixed VMAT strain, female WT mice showed elevated levels of consumption compared with other mice of that strain, and showed percent increase in excess of 100% for multiple sessions—this was the highest observed level of increase of any strain. Finally, the congenic VMAT BX strain showed similar effects as VMAT mice, but to a greater extent—higher levels of consumption and greater overall fluid consumption were observed in this strain, but the mixed VMAT strain showed the highest levels of escalation as measured in this study.

The present experiments examined the effects of reductions in the expression of the DAT and VMAT2 genes using two models of ethanol consumption. When evaluating the results of this study, it is important to consider what the expected changes in the DAT and VMAT2 KO strains would be based on what is known about dopaminergic and serotonergic function related to ethanol, and what roles DAT and VMAT2 have in modulating those systems. At the simplest level, deletion of DAT or VMAT2 would be expected to influence responses to ethanol based on the known effects of ethanol on DA neurons.

DAT KO and DAT BX Results

The present study found no genotypic effect on baseline ethanol consumption in either strain of the DAT KO mice. However, females in both strains showed higher consumption of ethanol, and mixed strain females also showed increased preference for ethanol solution compared with males, regardless of genotype. Previous studies of ethanol consumption and preference in strains of DAT KO mice have shown that female DAT mice do indeed consume more ethanol than males, independent of genotype (Hall et al., 2003; Savelieva et al., 2002). Ethanol treatment causes increased firing of dopaminergic neurons (Diana et al., 1993) and increases extracellular levels of DA and other monoamines in the NAc (Heidbreder & De Witte, 1993), which are both consistent with a role of DA in ethanol reward. Deletion of the DAT gene would be expected to increase DA actions by prolonging DA actions in the synapse, while VMAT2 deletion might be expected to have the opposite effect by reducing the amount of DA in vesicles (setting aside for the time the potential effects of VMAT2 deletion on other monoamines). However, when considering the roles of DAT and VMAT2 in the dynamic process of monoaminergic neurotransmission, distinct compensatory changes occur along with reduction of DAT in the DAT KO mice, including reduced DA synthesis (Jones, Gainetdinov, Jaber, et al., 1998), reduced autoreceptor function (Jones et al., 1999), and DA receptor levels (Sora, Hall, et al., 2001). Additionally, as suggested by Hall et al (2003), there may be a greater capacity for compensatory adaptations in heterozygous KO mice compared to homozygous mice, via receptor reserve (Sora, Elmer, et al., 2001) and other mechanisms, that might lead to opposite effects to that observed in homozygous KO mice. Such a compensatory change was seen in Hall et al. (2003), where heterozygous DAT KO female mice showed a significantly higher preference for ethanol and showed a trend towards increased ethanol preference, while

homozygous KO mice showed decreased preference compared to WT mice. While DAT and DA function may not be the only factor involved in the actions of ethanol and ethanol reward, deletion of DAT reduces termination of dopaminergic neurotransmission via reuptake, producing greatly elevated basal DA levels (Shen et al., 2006), as well as potentially increasing stimulated levels under some conditions. This allows more time for the DA that is released to interact with receptors on the postsynaptic cell and the distance at which DA is able to be an active neurotransmitter before inactivation by uptake or metabolic enzyme action.

VMAT and VMAT BX Results

Similar analysis of the effect VMAT2 KO reveals an even more complex interaction. VMAT2 is a vesicular membrane protein responsible for transporting monoamines from the cytosol into the vesicle, including both DA and 5-HT. Reduction of VMAT2 will reduce the amount of monoamine transport into the vesicles, ultimately reducing the amount of monoamines available for release. Research in heterozygous VMAT2 KO mice shows reduced tissue content of monoamines (Fon et al., 1997), extracellular monoamine levels (Wang et al., 1997), and the amount of monoamines released by depolarization or treatment with amphetamine (Wang et al., 1997). Reducing 5-HT and DA release by deletion of VMAT2 could result in competing effects. As previously discussed, reduced baseline serotonergic neurotransmission is associated with increased alcohol consumption (Rausch et al., 1991). Since deletion of VMAT2 has been shown to reduce monoamine levels (Wang et al., 1997), it follows that extracellular 5-HT levels would be reduced in VMAT2 mice. This could predispose VMAT2 KO mice to increased ethanol consumption because of the reduced 5-HT levels, as low 5-HT levels have

been linked with higher ethanol preference and consumption in rodents (Daoust et al., 1985). Alternatively, reduced DA release associated with overall reduced monoaminergic neurotransmission caused by VMAT2 KO after ethanol consumption might reduce ethanol consumption. The findings in the consumption studies are consistent with reduced DA release associated with the reduction of VMAT2 KO, although these effects were only seen in the congenic strain. Since congenic strains are bred to minimize the allelic differences at loci away from the actual knockout construct, this could suggest that the C57 background is important in revealing the effect of the KO. The congenic strains may be more likely to show the effects of KO—the elevated ethanol preference and consumption characteristic of congenic strains might model genetic factors that increase ethanol preference and consumption in humans. Therefore, the genetic background incorporates baseline changes that may be more susceptible to the effects of DAT or VMAT2 KO. Identifying interactions between the characteristic increased preference and consumption in congenic strains with specific gene KO could provide a useful animal model of polygenic interactions related to alcoholism.

In the first experiment mice had continuous access to ethanol and water for 24 hours a day (2 bottle choice continuous access), but differing concentrations of ethanol were presented in an ascending manner, from 2% to 32%. Under these conditions all groups of mice increased their consumption of ethanol solutions as the concentrations was initially increased from the lower concentrations of ethanol to moderate concentrations, and reduced consumption of ethanol solutions at the higher concentrations of ethanol (16% and 32%). DAT, DAT BX and VMAT BX mice showed increased preference and consumption in females, regardless of genotype. VMAT BX mice alone showed an effect of genotype, where heterozygous KO reduced ethanol preference and consumption, but further analysis showed that this effect was significant in males

only. This overall pattern was influenced by a variety of factors, including sex, background strain and genotype. In both strains of DAT mice, females consistently consumed more ethanol and showed higher preference for ethanol solution than males. For VMAT BX mice, a similar pattern of increased ethanol preference and consumption was observed for females. Interestingly, no effect of sex or genotype was observed for the mixed background VMAT KO mice.

Previous Studies of Ethanol Consumption in DAT and VMAT KO Mice

The present study found no genotypic effect on baseline ethanol consumption, measured in the first experiment, related to either strain of the DAT mice, however, females in both strains showed higher consumption of ethanol, and mixed strain females also showed increased preference for ethanol solution compared with males, regardless of genotype. Previous studies of ethanol consumption and preference in strains of DAT KO mice have shown that female DAT mice do indeed consume more ethanol than males, regardless of genotype (Hall et al., 2003; Savelieva et al., 2002) under similar conditions. Hall et al. (2003) found no genotypic difference between DAT males, but did find female hKO mice showed increased ethanol preference, particularly at lower concentrations. As previously mentioned, methodological differences can influence whether or not genotypic differences are observed in transgenic mouse strains. Hall et al. (2003) presented concentrations for two to three days, but used more intermediate concentrations. Savelieva et al. (2002) did not use concentrations higher than 15%, but did present each concentration for six to eight days each. Savelieva et al. (2002) found no difference between heterozygous KO and WT female mice, but noted a decrease in ethanol consumption

and preference in homozygous KO female mice. As this study only used WT and heterozygous KO mice, these results seem to be in line with one another. One limitation of the present studies is that only WT and heterozygous KO mice were used—no homozygous KO mice were used. The VMAT2 homozygous KO is lethal, so it is not possible to examine the differences in ethanol consumption between hetero- and homozygous KO of VMAT2. However, both Savelieva et al. (2002) and Hall et al. (2003) used homozygous DAT KO mice in addition to the heterozygotes. It could be that heterozygous KO does not produce enough of a change to the monoaminergic systems to affect ethanol consumption in a genotypic-dependent manner.

Previous studies of ethanol consumption in VMAT and DAT KO mice have produced conflicting results, which was part of the impetus for these studies. Savelieva et al. (2006) found that female mice drank more ethanol solution than males in a two bottle choice paradigm, which is consistent with the results of the present research. Savelieva et al. (2006) also found that VMAT hKO males showed decreased preference for and consumption of ethanol solution compared to WT mice on a mixed genetic background. The present study found no effect of genotype in the mixed background VMAT strain, but in the congenic VMAT strain, a similar decrease in preference and consumption in male hKO mice was observed. When mixed genetic backgrounds are made in producing transgenic mice in this manner, the alleles from the parental strain fixate within a short period of time on one of the parental alleles (this is in part due to the generally low number of breeding pairs in any laboratory population). Thus, it is highly likely that the particular alleles in the two strains of transgenic mice used in these studies fixated on different parental alleles and, consequently, the Savelieva strain is more similar to the congenic VMAT2 strain because it contains more C57 alleles that influence ethanol consumption. One distinct difference between Savelieva et al. (2006) and the present study is the difference in

genetic background between the mice used in the study. We know that factors contributing to alcohol consumption and abuse are highly polygenic. The present study replicated the same sex and genotypic effects in the congenic strain, compared with the original findings on a mixed background, suggesting that there is some genetic influence in the mixed background that attenuates the effect of the VMAT KO. Hall et al. (2003) also examined the role of VMAT KO on ethanol consumption in a two bottle choice paradigm. The same sex effect previously described was also observed in that study. However, Hall et al. (2003) observed an increase in ethanol preference and consumption in male hKO mice at higher ethanol concentrations (16%, 24%, and 32%). The highest concentration used by Savelieva et al. (2006) was 15% ethanol, which might account for the failure to observe the same effects in that study. However, the present study did test higher concentrations (16% and 32%) of ethanol and did not observe this effect. Both Hall et al. (2003) and Savelieva et al. (2006) presented each concentration for longer periods of time—2-3 or 6-8 days, respectively, while the present study only presented each concentration for two days. This shorter duration of exposure might have contributed to the failure to observe the differences observed in the previous studies.

Importance of Method in Revealing Genotypic Difference in Ethanol Consumption

It has previously been observed that the contributions of different genetic effects to ethanol consumption can be highly paradigm dependent. When inbred and hybrid mouse strains were compared in multiple experiments comparing ethanol consumption, a two-bottle choice test showed increased ethanol consumption and preference in C57BL/6J mice compared with all other inbred and hybrid strains. However, when ethanol consumption was measured in limited

access (4 hours, 3x/week), strains consumed similar amounts of ethanol, and analysis of average consumption for all sessions showed that a hybrid strain (C57BL/6J × 129S4/SvJae) consumed more than the standard C57BL/6J strain (Lim, Zou, Janak, & Messing, 2012). Another example of the importance of method used to access ethanol consumption comes from a study characterizing ethanol consumption in a metabotropic glutamate receptor 5 (mGluR5) KO mouse strain, the authors used five different consumption protocols to assess ethanol preference and consumption (Blednov & Harris, 2008). Blednov and Harris (2008) only observed genotypic differences in two of the five consumption paradigms. This suggests the importance of method in elucidating effects of gene KO on ethanol consumption. Blednov and Harris (2008) used the same strain of mGluR5 KO mice, in the same laboratory, under the same conditions, and saw varied genotypic differences dependent on method. Lim et al. (2012) challenge the labeling of rodent strains as "ethanol-preferring" or "ethanol non-preferring" without appropriate consideration of method in determining preference and consumption in these strains, because as seen in previous research, differences in method can determine whether or not differences in ethanol consumption are observed. Therefore, when determining the effect of specific gene manipulations on ethanol consumption, any observed differences in ethanol consumption based on genotype need to be evaluated within the context of the methods used to access ethanol consumption. In terms of evaluating the present results with respect to previous research, additional differences of strain and method present between Savelieva et al. (2006), Hall et al. (2003), and the present study certainly contribute to some of the differences seen between the three studies. Based on this comparison, it would appear likely that the failure to observed consistent genotypic differences in DAT KO and VMAT KO mice may result from a failure to identify the optimal paradigm in which these genes contribute to ethanol consumption.

Alternatively, ethanol consumption in this study was limited to 10 days. It could be that the relatively short-term ethanol consumption in this study is more reliant on DA neurotransmission than 5-HT neurotransmission. Previous research supports interactive yet distinct effects of ethanol on 5-HT and DA. For example, examination of ethanol consumption in ethanol preferring, non-preferring, and neutral strains showed that ethanol-preferring strains showed reduced extracellular 5-HT levels in ethanol preferring rats, but no differences in basal DA release. The authors suggest that these changes may contribute to genetic determination of ethanol preference and consumption (Smith & Weiss, 1999). Alternatively, there are clear interactions between the serotonergic and dopaminergic systems in response to ethanol, such as 5-HT mediated DA release through 5-HT_3 receptors (A. D. Campbell et al., 1996; Wozniak et al., 1990). Further research is necessary to determine whether the effects observed in this study are related more to DA than 5-HT.

Compensatory Changes Associated with Heterozygous KO

As suggested with the DAT KO mice, it has been suggested that heterozygous KO mice maintain more capacity for normalization of function than homozygous KO mice (due to available receptor reserves). There is, therefore, the possibly that heterozygous deletion of these genes may not be enough to produce genotypic differences between the mice because of issues of receptor reserve. Since the VMAT2 KO has the potential to affect multiple monoaminergic systems, the compensatory changes in heterozygote KO could mask the effects of the KO. In other words, the changes caused by the KO alone (reduced monoamine neurotransmission) could be masked by changes in other parts of monoaminergic systems, such as reduced autoreceptor

function (Jones et al., 1999), and DA receptor levels (Sora, Hall, et al., 2001) in DAT KO mice. In fact, VMAT2 KO has been shown to reduce DAT levels in mice (Yamamoto et al., 2007), and increase activity of the 5-HT $_{1A}$ autoreceptor (Narboux-Neme et al., 2011). Homozygous KO of VMAT2 is lethal in mice, so the chance to evaluate hetero- versus homozygous KO is not an option. Further research investigating the effects of acute versus chronic ethanol consumption could provide insight into these questions.

Escalation Method

The escalation paradigm used in this study tested whether or not mice would increase their ethanol consumption and/or preference when allowed access to both ethanol (8% v/v) and water for 24 hours at a time intermittently, twice per week. All groups escalated their consumption of ethanol solution (volume), ethanol per unit body weight (g/kg/day) and preference. DAT and VMAT2 mice consistently showed significant sex differences, where female mice consumed more ethanol and had higher ethanol preference than male mice, regardless of genotype. Interestingly, in DAT mice, male mice escalated at a greater rate than female mice. At session six, the final session in the experiment, female hKO mice had escalated to similar levels as the male mice, but WT females remained lower than males. As females had overall higher ethanol consumption (approximately 1 gram higher than males for session one) at the beginning of the paradigm, this effect could represent ceiling effects because of the high initial ethanol consumption in females (e.g. they could be thought of as “pre-escalated”). The females would have to consume more ethanol than the males to produce the same percent change in consumption. At the moderate (8% v/v) concentration of ethanol solution used in this

experiment, it could be that it took the females longer to increase their consumption because it required adaptations associated with drinking a lot more fluid in order to increase ethanol consumption. While all groups escalated their consumption, analyzing the individual difference from the group mean (percent difference) revealed interesting patterns in the escalation of the two congenic strains. Both congenic strains showed an interaction of SESSION x GENOTYPE for ethanol consumption expressed as g/kg/day. DAT BX mice showed initial consumption at 8-9 g ethanol consumed per kilogram body weight per day. Male hKO mice increased to about 10.5 g/kg/day, but all other groups increased consumption to about 14 g/kg/day. In both strains, heterozygous KO reduced percent escalation and there was a direct effect of GENOTYPE in the ANOVA. For DAT BX mice, WT mice escalated about 80% from the initial measurement by session four, while KO females escalated an average of 50% by session six and KO males escalated the least to 30%, which stabilized by session four.

To summarize, there were two main findings in terms of genotypic effects of manipulation of the DAT and VMAT2 genes. First, only congenic strains differed in this measure dependent on genotype. Second, heterozygous KO of both the DAT and VMAT2 genes produced reduced escalation of ethanol use. While the results of the consumption experiments might suggest that heterozygous KO of DAT or VMAT2 may not produce enough of an effect on the monoaminergic system to affect ethanol consumption, the escalation experiments show support two conclusions. First, the data support that heterozygous KO of both genes can affect ethanol consumption and escalation and therefore the heterozygous deletion produces sufficient changes to change ethanol consumption. Second, the present data support the findings that suggest method used to access ethanol consumption in transgenic mouse models may be of vital

importance when trying to determine the effects of gene manipulation on ethanol consumption and escalation.

Behavioral Models Used in Alcohol Research

Behavioral animal models are developed and used to represent symptomology and behavior thought to be important in a given disorder. One of the key diagnostic criteria for alcohol dependence, according to the Diagnostic and Statistical Manual of Mental Disorders (4th ed. text revision, American Psychological Association, 2000). Determining the conditions under which the transgenic mouse model increases ethanol consumption, particularly voluntary ethanol consumption in a two-bottle choice test, could provide a behavioral model of this feature of alcoholism. However, escalation of alcohol use is a specific diagnostic characteristic of alcoholism. In determining whether or not the model for escalation presented in this study models symptoms of alcoholism, reasons why animals escalate must be considered. In a review of animal models used in alcohol related research, Tabakoff and Hoffman (2000) identify the face validity of animal models that identify alcohol seeking behavior, which animals engage in because of the reinforcing effects of ethanol. However, in all animal models, researchers are in fact providing ethanol, either through direct administration (injections) or by providing access to ethanol (two-bottle choice). Because of this, animal models are limited in their ability to represent ethanol (or other drug) seeking behavior and other motivational aspects of alcohol abuse. Of all animal models of alcohol-related disorders, two-bottle choice paradigms have higher levels of face validity because of the similarities of increased ethanol consumption when ethanol is presented with sweetened solution and the overall range of differences in baseline

ethanol consumption between different strains of mice. Two-bottle choice paradigms have simple face validity in that mice can choose how much (or little) ethanol to drink, but this model does not include other aspects of voluntary ethanol consumption related to alcohol abuse, such as escalation of alcohol consumption. The present study attempted to design a behavioral model in which mice voluntarily (through two-bottle choice) escalated ethanol consumption. This model has face validity in that the mice voluntarily chose to escalate consumption of an ethanol solution for which they showed high initial preference. This model of escalation has the potential for a degree of predictive validity as well—if manipulations of the DAT and VMAT2 genes can cause changes in escalation of alcohol consumption, a key feature of alcoholism. This model can then be used to identify how genetic manipulations, and potentially pharmacological interventions, involving DAT and VMAT2 affect escalation of ethanol consumption. The results support that DAT and VMAT2 manipulation change ethanol preference, consumption, and escalation, as seen in this study, particularly the results of the escalation study. The fact that ethanol escalation is a direct symptom of alcoholism in humans, the model presented in this study has the potential to be useful in further research of the roles of DAT and VMAT2 in the underlying neurobiological mechanisms of and treatment for alcohol-related disorders.

Aside from the behavioral measures and models, the present study attempted to present transgenic mouse models of conferred risk to escalate alcohol consumption. While the genotypic differences in this study are in opposition to the proposed effects of DAT and VMAT2 KO (KO would increase ethanol consumption and preference), the results serve to identify circumstances where genotypic differences are observed. Moreover, since specific genetic differences tend to be associated with subsets of alcoholism (i.e. DAT 3' UTR polymorphism associated with withdrawal seizures and delirium), identifying genotypic differences in the DAT and VMAT2

KO strains helps identify what roles these transporters play in the complex disorder of alcohol dependence. The results from this study showed reduced escalation in the heterozygous KO mice. As previously suggested, this could be the result of compensatory changes related to the heterozygous KO, an interaction between the KO and background strain (specifically how much of the C57 background became incorporated in background through breeding the KO strain) a result of the relatively short method used, or a combination of these factors. Further research comparing acute and chronic consumption in these KO mice could help clarify the roles of the heterozygous KO of DAT or VMAT2 as transgenic models for alcoholism. Congenic heterozygous KO mice had reduced consumption and percent escalation in the escalation paradigm. C57 mice are known to have increased consumption compared with mixed C57/129 mice. In the present experiments, interaction of ethanol preferring background combined with DAT or VMAT2 heterozygous KO produced reduced ethanol consumption and escalation of use. This means that manipulations that reduce DAT or VMAT2 expression or activity, particularly in individuals that show increased preference and consumption of alcohol (i.e. individuals with alcohol dependence), might treat alcoholism.

Heterozygous KO as a Model of Human Genetic Variation

A main motivation in using heterozygous KO mice in this study was to more closely model the levels of variation of expression of these genes in humans, and therefore have a more direct relevance to the study of alcoholism. The review presented above describes many of the allelic variation of the DAT gene in human populations, which are putatively associated with alcoholism mainly through the modifications to the dopaminergic reward circuit. These allelic

variations include association of the sequence variants of DAT with increased risk for alcoholism (Higuchi et al., 1994; Lin & Uhl, 2003), 3'-UTR polymorphisms associated with withdrawal seizures and delirium (Le Strat et al., 2008; Sander, Harms, Podschus, et al., 1997; Ueno et al., 1999), and associations between occurrence of the 7-repeat allele (Dobashi et al., 1997; Muramatsu & Higuchi, 1995) and 9-repeat allele (Dobashi et al., 1997; Samochowiec et al., 2006; Sander, Harms, Podschus, et al., 1997) with alcoholism. As VMAT2 also is able to modulate DA neurotransmission, the role of VMAT2 in ethanol reward and abuse, treatment of alcohol related disorders, or some combination of the two is important in further research. The current observations of method-, sex-, and genotype-dependent differences in baseline ethanol preference, consumption, and escalation supports a role of dopaminergic influences on ethanol consumption. While complex, these findings are consistent with the complex polygenic determination of alcoholism. Due to these different factors, treatments for alcoholism may be dependent on the difference allelic contributions to alcoholism. The present findings motivate research investigating the methods under which these genes have a more prevalent role in modulating ethanol consumption, and research understanding how the sex dependent effect observed consistently in DAT and VMAT2 mice relate to risk for alcohol related disorders in human populations.

Future Directions

A main motivation of the escalation experiment was to identify parameters under which DAT and/or VMAT2 modulated ethanol preference, consumption, or escalation. The present experiments found both a paradigm under which all strains voluntarily escalated consumption,

and identified conditions under which genotypic differences were seen in ethanol consumption and escalation. Using this experimental paradigm, three pharmacological agents will be tested for their efficacy in increasing DAT and VMAT2 expression—Puerarin, Dl-3-n-Butylphthalide (NBP), and edaravone. NBP has been shown to increase expression of VMAT2 *in vivo* and *in vitro* (Xiong et al., 2012). Similarly, edaravone has also been shown to increase levels of VMAT2 expression (Xiong et al., 2011). Effects of treatment with each of these drugs, which are known promoter up-regulators of DAT and VMAT2, will be evaluated using quantitative reverse transcription PCR (rtPCR) to measure levels of expression of DAT and VMAT2, and related to behavioral effects of treatment with these drugs in the escalation paradigm. Thus the present experiments have identified conditions under which specific contributions of DAT and VMAT2 to alcoholism can be tested in animal models, and in which it might be possible to relate specific treatments to specific underlying genetic bases.