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Changes in AUTS2 Expression and Histone Dopaminylation Levels in High Ethanol Consumption Models

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May 2023

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Abstract

Developing efficacious treatments for alcohol use disorder (AUD) requires an understanding of the disorder on a genetic, molecular, and behavioral level. It is a complex and multifaceted disorder with many intrinsic and environmental factors, which means that it can be difficult to treat. Recent research has identified several genes potentially involved in the development and progression of AUD, including *Autism susceptibility candidate 2*, or AUTS2. AUTS2 is a gene that is linked to a number of neuropsychiatric diseases as well as alcohol addiction. This study aims to further characterize the involvement of AUTS2 as a modulator of alcohol consumption in AUD phenotypes, using mice as a model system. Additionally, a novel form of epigenetic regulation, histone dopaminylation, was investigated in an attempt to determine its role in AUD. Histone dopaminylation has recently been linked to addiction-like behaviors and cocaine-seeking in rats, but the presence and impacts of this modification in alcohol models have not yet been explored. Comparisons were drawn between mice that have a history of ethanol (alcohol) consumption and animals that have no history of consumption, as well as between animals with differing levels of alcohol consumption. The results of the study reveal differences in AUTS2 expression between wildtype and heterozygous AUTS2 knockout animals in different regions of the adult mouse brain. Some regions of the brain, such as the nucleus accumbens, showed increased AUTS2 expression in heterozygous animals, who drank more alcohol on average than their wildtype counterparts, while other brain regions showed no significant differences or increased expression in the wildtype animals. Additionally, animals with no history of alcohol consumption showed significantly less AUTS2 expression in certain brain regions when compared with animals with a history of consumption. Unfortunately, the experiments involving histone dopaminylation revealed no results that were able to be validated, potentially due to issues with antibody optimization. However, the results of this study

demonstrate a potential link between alcohol consumption and AUTS2 expression in adult mice and may help guide future studies into AUTS2 and its role in AUD.

Introduction

Alcohol use disorders (AUD) are extremely prevalent across the globe. In the US alone, AUD affects 29.5 million people over the age of 12 (SAMHSA, 2021) and accounts for nearly 140,000 deaths (CDC, 2022). It is a complex disorder that is difficult to treat, with a variety of genetic and environmental factors. To develop effective treatment for AUD, it is vital that we understand the disease on a molecular and genetic level. AUD has significant genetic underpinnings, which has been a major focus of study in recent years. We recognize that our genes play a role in the development and progression of chronic alcohol drinking. For this reason, it has been a goal of many studies in recent years to identify genes that contribute to the propensity to drink.

One of the first studies to take major steps towards this goal identified 36 candidate genes for preference drinking in a two-bottle choice paradigm in mice that also overlapped with alcoholism susceptibility in humans using a whole-genome meta-analysis (Mulligan et al., 2006). The study also investigated several different mice strains that differ in their alcohol preference and used them to show that pathways regulating transcription, and therefore gene expression, could play an important role in establishing high levels of voluntary alcohol drinking in mice (Mulligan et al., 2006).

One such gene that has been identified is *Autism susceptibility candidate 2*, or *AUTS2*. *AUTS2* was first identified in a study surrounding a monozygotic twin pair with autism and was hypothesized as the gene responsible for the twins' disorder (Sultana et al., 2002). The gene has also been linked to attention-deficit hyperactivity disorder (ADHD) and has suggested links to impulsivity (Elia et al., 2010), which could be relevant in both ADHD and alcohol addiction. Studies into this gene have found that it is expressed in a variety of brain regions, including the hippocampus, prefrontal cortex, and cerebellum (Bedogni et al., 2010) as well as regions

involved in alcohol reinforcement such as the ventral tegmental area and nucleus accumbens (Schumann et al., 2011). *AUTS2* has also been found to be expressed in dopaminergic neurons and frontocortical glutamatergic and GABAergic neurons, which could further indicate the gene's role in alcohol reinforcement and sensitivity (Schumann et al., 2011). A genome-wide association study on daily alcohol intake revealed several convincing arguments for *AUTS2*'s involvement in AUD. Firstly, differential *AUTS2* expression was seen in whole-brain extracts of seven mouse models known to have a high level of voluntary drinking (Schumann et. al, 2011). Additionally, *AUTS2* is found on a gene locus known to be involved in alcohol preference (Schumann et al., 2011). In humans, a single-nucleotide polymorphism (SNP) in *AUTS2* was associated with alcohol consumption. Finally, a study of the *AUTS2* homolog in *Drosophila* (fruit flies), *tay,* showed that down-regulation of *tay* induced decreased alcohol sensitivity, further supporting *AUTS2*'s involvement in alcohol preference (Schumann et. al, 2011).

In addition to identifying key genes (such as *AUTS2*) that may be involved in alcohol use disorder and contribute to the propensity to drink and investigating how the expression of these genes is influenced by alcohol consumption, it is also important to determine how larger gene networks might play a role in the development and progression of AUD. An emerging theme is that epigenetics, or changes in gene function caused by behavioral or environmental influences, play a significant role in the alcoholic brain. Altered transcription and gene expression in AUD is largely driven by epigenetic regulation in the form of DNA methylation, histone modifications, and chromatin remodeling. Epigenetic changes to our genome may be caused by alcohol consumption via the activation or suppression of chromatin. Changes in the way chromatin is structured are associated with changes in activation or repression of transcription, which may in

turn dramatically alter gene expression. Altered gene expression in the brain could lead to many of the molecular and behavioral changes seen in AUD.

AUTS2 has been identified as a potential key epigenetic regulator of alcohol consumption (Schumann et al., 2011). Interestingly, AUTS2 has been found to serve a non-canonical role in the protein complex with which it associates (polycomb repressive complex 1, or PRC1). These protein complexes normally operate by maintaining a repressive form of chromatin, thereby stimulating gene repression through epigenetic mechanisms (Gao et al., 2014). However, instead of repressing transcription, PRC1-AUTS2 activates transcription through recruitment of protein kinase CK2 and histone acetyltransferase P300, (Gao et al., 2014), suggesting that AUTS2 may impact normal transcriptional processes. However, despite *AUTS2*'s association with alcohol use disorder, the direct role of the PRC1-AUTS2 association on gene expression in the brain is poorly understood.

Research in the Stafford Lab on *AUTS2* and its role in AUD focuses broadly on how AUTS2 drives transcriptional and behavioral changes in mouse alcohol consumption models. Previous projects in the lab have investigated how AUTS2 may influence AUD-related phenotypes and behaviors. The aim of the present study within the framework of the larger body of work in the lab is to investigate whether there are significant molecular changes, namely, in the expression of *AUTS2,* associated with differing levels of alcohol consumption. This work aims to further characterize the expression profile of *AUTS2* in AUD mouse models, looking at how AUTS2 changes in response to alcohol consumption.

Additionally, the study aimed to identify and characterize an epigenetic marker in mice that drink high volumes of alcohol and determine how epigenetic signatures might change in response to varying amounts of alcohol consumption. The epigenetic marker that was considered

in this study is histone dopaminylation, a novel histone modification. Histones are the main protein component of chromatin, serving as a spool for DNA to wrap around into a compact form. Like other histone modifications, histone dopaminylation involves post-translational modification of histone proteins through the association of different functional groups or neurotransmitters with chromatin, changing its structure. In this case, the neurotransmitter dopamine is covalently attached to histones. A recent study has established the role of histone dopaminylation (the covalent linkage of dopamine to proteins) in addiction-like behaviors in rats (Lepack et al., 2020). Accumulation of dopaminylated histones was seen in rats undergoing cocaine withdrawal (Lepack et al., 2020). Additionally, reducing levels of dopaminylated histones resulted in the reversal of cocaine-mediated gene expression and reduced drug-seeking behaviors (Lepack et. al, 2020). Histone dopaminylation's recently established role in addiction makes it an interesting avenue of research for this study. Because this is a newly discovered modification, it is not currently known how alcohol consumption and withdrawal impacts histone dopaminylation and vice versa. An initial goal of this research was to understand how this histone modification is impacted by alcohol consumption.

Mice and other rodents have long been a useful tool in studying AUD and other addiction-like behaviors, as evidenced by many of the studies described above. There are an abundance of strains and tools available for rodent models. Many important discoveries have been made possible using alcohol-specific mouse strains such as C57BL/6J mice, which voluntarily consume alcohol and consume a significantly higher volume of alcohol than other strains (Lê et al., 1994). High alcohol preferring and low alcohol preferring (HAP/LAP) mice are frequently used in AUD studies—these mice were created by 10 generations of selection for differences in two-bottle choice alcohol consumption (Grahame et al., 1999). HAP mice were

used in this study to look at differences in AUTS2 expression in high-alcohol-preferring mice who have a history of alcohol consumption versus mice who have no history of alcohol consumption. Another mouse model that was important in this study was an AUTS2 knockout (KO) model, A7NesCre, which used CRE recombinase driver lines to delete AUTS2 from the whole brain. The cohort consisted of wildtype (WT), heterozygous (Het), and full KO animals, but only the WT and Het animals were used in this study.

Firstly, it was important to validate *AUTS2* and histone dopaminylation expression in the WT AUTS2 animals. This was done over the first several months of the project and included a number of experiments and troubleshooting endeavors to ensure that AUTS2 and dopaminyl staining was visible and optimized in the WT animals. The antibody used for histone dopaminylation detection, Anti-H3K4me3Q5dopaminyl, has not previously been used for IHC, only western blot and other more quantitatively sensitive techniques. While western blot has the advantage of generating a signal proportional to the amount of protein in a sample (Moore, 2020), IHC provides the advantage of seeing localization of AUTS2 and dopaminylation in situ. Therefore, optimization was required to make both the AUTS2 and dopaminyl IHC stains more viable.

One optimization technique that was added to the protocol to improve signal detection is antigen retrieval, which is a technique that is used to increase the binding affinity of a primary antibody to its antigen and has been proven a highly effective method for rendering IHC staining (Shi, Shi, and Taylor, 2011). Further optimization techniques includes adjusting antibody concentrations and implementing another method of staining, immunofluorescence (IF).

Both WT and Het AUTS2 KO animals from the A7NesCre cohort were used in this study to investigate *AUTS2* expression in mice for two reasons. The first was to compare expression

levels between WT and Het, and the second was to compare between different levels of alcohol consumption. We used IHC to assess *AUTS2* expression and histone dopaminylation in these animals in several brain regions of interest, including the cortex, hippocampus, and nucleus accumbens. We know from previous studies that *AUTS2* expression is prominent in these areas and thus were the areas selected for analysis.

Another experiment was also added to the study to investigate differences in AUTS2 staining at different consumption levels. A cohort of HAP mice was used to examine differences between animals with a history of alcohol consumption and animals with no history of alcohol consumption, as half of the cohort drank alcohol and the other half drank only water. Similar to the previous experiment, IHC was used to assess *AUTS2* expression in these animals. It was hypothesized that the animals that drank EtOH as opposed to H2O would show increased *AUTS2* expression in the brain regions of interest.

Both experiments described above were conducted in the hopes of gaining valuable insight into AUTS2 expression in the mouse brain and how it is affected by alcohol consumption. Gaining a better understanding of *AUTS2* and its expression patterns in the brain can help to further our knowledge and understanding of how our genes change in AUD. Additionally, comparing between different levels of alcohol consumption could elucidate and clarify the associations between increased *AUTS2* expression and high levels of drinking. Understanding these changes may reveal potential predictors of AUD, as well as guide diagnosis and targeted therapeutic interventions for individuals with AUD.

Methods

Animals

The animals used in this study were used to both validate *AUTS2* expression and histone dopaminylation in the mouse brain as well as compare between animals with differing levels of alcohol consumption and/or no history of alcohol consumption. The first group of mice used was a cohort of AUTS2 whole-brain knockout animals, A7NesCre. The cohort contained WT, Het, and KO animals. The animals used in this study were the WT and Het animals, which were first used to confirm the presence of AUTS2 staining using the IHC protocols described later in this section. The WT animals were initially hypothesized to exhibit more AUTS2 staining than the Het animals, as the Het animals only carry one copy of the *AUTS2* gene. These animals were later used to compare between differing levels of alcohol consumption, as it was found during their intermittent access drinking regimen (described in the next section) that the Het animals drank more than the WT animals, which might lead to increased *AUTS2* expression and increased histone dopaminylation levels. The second group of animals used in the study were high-alcohol-preferring (HAP) mice, which have been selectively bred to drink large amounts of alcohol. The HAP cohort underwent a continuous access two-bottle choice model, with half of the cohort receiving access to both H2O and 10% ethanol every day, while the other half of the cohort received only H2O. These animals were used to compare between animals that have a history of drinking alcohol and animals that have no history of alcohol consumption.

Intermittent and Continuous Access Models

Drinking was used in this study as a tool to affect the brain. Other projects in the Stafford Lab have examined the behavioral effects of alcohol drinking in the mice cohorts described

above, but this project focuses on the molecular impacts of alcohol consumption on the brain. One of the most common models used to simulate heavy levels of alcohol consumption in mice is the continuous access model, which provides mice with a two-bottle choice paradigm with one bottle containing 10% ethanol and the other containing water. While continuous access models are among the easier and less invasive models to study alcohol consumption (Bloch 2022), the intermittent access two-bottle choice provides another efficient way to model alcohol consumption in mice. The intermittent access model differs from continuous access models in that animals are given access to ethanol only every other day and the concentration of ethanol is increased to 20%. This method of access causes mice to escalate their drinking very quickly and it has been shown that mice in the intermittent access model also tend to show significantly higher ethanol preference than mice in the continuous access model (Hwa et al., 2011). The escalating, voluntary ethanol consumption seen in intermittent access mice makes it a good model for studying AUD. The intermittent access model was implemented for the A7NesCre cohort, which underwent approximately 1 month of drinking. These drinking experiments were completed in an affiliated mouse facility that helps ensure animal welfare standards are upheld. The mice were euthanized immediately after cessation of alcohol access and brains were harvested for immunostaining.

Fixation and Cryoprotection

Following euthanization and tissue harvest, whole brains were placed in tubes containing paraformaldehyde (PFA) and put on ice for fixation. The brains were left in the PFA solution for 24 hours to ensure that the structure and morphology of the cells and tissues was preserved and to protect the tissue during subsequent processing.

Before the brain can be sliced, it must go through cryoprotection, which protects tissue from freezing damage. Following fixation with PFA, the brain was removed from PFA and placed in a solution containing sucrose (a cryoprotectant) and sodium azide.

Slicing and Cryostat Use

After the brains were fixed and cryoprotected, cryosectioning was performed using a cryostat at around -22°C. After the brain is frozen, it can then be sectioned into 30-micron thick slices. First the brain was carefully mounted onto a chuck, which holds the brain in place along with the use of optimal cutting medium (OCT), a clear, viscous liquid that freezes quickly and turns opaque when it is fully frozen. A dollop of OCT was placed on the chuck and allowed to freeze for about a minute to create a mount. Coronal sections were used in my project, so a small cut in the cerebellum was made using a razor blade so that the brain can sit upright on the chuck. Another small dollop of OCT was added to the existing OCT mount, then the brain was carefully transferred into the OCT to attach the brain to the chuck. It is important to make sure the brain is placed as straight as possible on the chuck so that the left and right hemispheres of the brain are as even as possible when slicing. After attaching the brain to the chuck, a thin layer of OCT was added to coat the entire brain and the brain was allowed to freeze within the chamber of the cryostat for about an hour before slicing.

Slicing is done within the chamber of the cryostat using a very thin blade. After the brain is frozen, the chuck is secured in the cryostat mount and brought in line with the blade. A lever is used to control the position of the chuck and create 30 micron-thick slices, which were collected on a metal platform in front of the blade. When slicing, it is important to ensure the slices are even, which can be done by looking for landmarks in the brain. Slices collected on the platform were then transferred with a paintbrush into 12 well plates containing 0.1% Sodium azide in PBS

solution, which was the storage buffer used to store the slices. After the whole brain is sliced, parafilm was used to cover the plates to prevent evaporation and spilling, and the slices were stored in the fridge until immunohistochemistry was performed.

Antigen Retrieval, Immunohistochemistry, and DAB

Immunohistochemistry (IHC) was used to stain the samples, which allowed for visualization of AUTS2 and histone dopaminylation expression. Vector Laboratories ImmPRESS HRP Horse Anti-Rabbit IgG Polymer Detection Kit was used for antibody staining for this study. Slices stored in 0.1% Sodium azide in PBS were first mounted on slides using a paintbrush and excess PBS and were allowed to dry before continuing with the immunohistochemical staining protocol.

Before beginning the ImmPRESS protocol, an additional step was added to the staining protocol to ensure visualization of the antigens--antigen retrieval. The protocol used in this study involves using an antigen unmasking solution in combination with a high temperature treatment procedure. Vector Laboratories Antigen Unmasking Solution was used in this study to perform the antigen retrieval step. The protocol provided by Vector Laboratories involves the use of a pressure cooker to expose the tissue to high temperatures, but as a pressure cooker was not available for use in this project, the protocol was adjusted to use a heat block instead. The mounted slices were first washed in water. Antigen unmasking solution was prepared using 10 µL of concentrated stock antigen unmasking solution and 990 µL of double-distilled water. The diluted unmasking solution was transferred onto the mounted slices, then the slides were placed on a heat block at 120°C for 1 minute, and immediately washed in water, ensuring that the sections do not dry out. The slices were then washed in PBS.

Following the antigen retrieval step, the ImmPRESS protocol was followed, beginning with a wash in BLOXALL Blocking Solution, followed by incubation with 2.5% normal horse serum, then incubation with primary antibody solution overnight. The antibodies used in this study were Sigma Aldrich Anti-AUTS2 antibody produced in rabbit and Millipore Sigma Aldrich Anti-H3K4me3Q5dopaminyl antibody produced in rabbit. Antibody solutions were prepared using 0.1% Triton-X in PBS, diluted normal horse serum, and antibodies at their indicated concentrations. For anti-AUTS2, a concentration of 1:200 was used, and several concentrations of anti-dopaminyl were used to determine the optimal concentration, including 1:500, 1:250, and 1:100. After incubation with primary antibody solution, the sections were incubated in ImmPRESS Polymer Reagent for 30 minutes, followed by 2 PBS washes.

The Vector Laboratories ImmPACT DAB kit was used for signal detection following incubation with the polymer reagent. The addition of DAB (diaminobenzidine) to a section that has undergone IHC results in the formation of a brown-colored precipitate at the location of antibody binding (Pawliszyn, 2012). The DAB solution was prepared using ~30 µL of ImmPACT DAB reagent and 1 mL of ImmPACT DAB Diluent. Slices were incubated for approximately 2.5 minutes with the DAB substrate working solution until the desired stain intensity developed. Optimal developing times were determined from earlier experiments and by looking for the slices to develop a light brown color. After incubation with the DAB solution, the slices were washed in water and then coverslipped with VectaMount aqueous mounting medium.

Immunofluorescence

Immunofluorescent staining was performed for visualization of dopaminylated histones As previous attempts at IHC for this antibody showed no true staining, this new method of staining was tested. Similarly to the IHC protocol, slices were mounted on slides and underwent

antigen retrieval prior to beginning the staining process. Sections were first incubated in a blocking solution comprised of 0.1% Triton-X in PBS and 2.5% normal goat serum (NGS) for 1 hour. Sections were washed in PBS and then primary antibody was added to the sections, which were then incubated in the primary solution overnight. Primary antibody solution consisted of 0.1% Triton-X and 1% NGS in PBS, with an Anti-H3K4me3Q5dopaminyl concentration of 1:250. The next morning, sections were rinsed in PBS. During the remaining steps of the protocol, the sections were light-protected, as the secondary fluorescent antibodies are sensitive to light. The sections were incubated in a secondary antibody solution, comprised of 1:1000 anti-rabbit conjugated 488 (green) secondary antibody in PBS with 0.1% Triton-X and 1% NGS. Following incubation, the sections were once again washed in PBS and then coverslipped with VectaShield HardSet Antifade Mounting Medium with DAPI. The slides were left to dry overnight in the laboratory chemical fume hood. Images were obtained from the slides the following day.

Imaging and Data Analysis

Imaging of the sections was performed using a BioTek Lionheart FX Automated Microscope. Images were taken from 4 WT A7NesCre slices in the nucleus accumbens region, 4 WT A7NesCre slices in the hippocampal region, 4 Het A7NesCre slices in the nucleus accumbens region, 4 Het A7NesCre slices in the hippocampal region, and two slices from the HAP cohort, one from an animal that consumed EtOH and the other from an animal than consumed only water. In total, n = 4 WT A7NesCre animals, n = 4 Het A7NesCre animals, and n = 2 HAP animals (one from each group) were assessed. The brain regions of interest (cortex, nucleus accumbens, hippocampus) were visualized using the microscope and selective images of these regions were taken for analysis. Images of the cortex were taken from the slices in two

different regions: dorsal to the nucleus accumbens (anterior cortex) and dorsal to the hippocampus (posterior cortex). In order to observe and determine differences in the number of AUTS2 positive nuclei and dopaminylated histones, ImageJ was used to count positive nuclei using a cell counter plugin within ImageJ. Using this plugin, 125 x 125 μm sections were chosen from the images and the number of positive cells were counted. Uniform brain areas were sampled to ensure that the data is representative of actual differences in AUTS2 expression and dopaminylated histones levels in the various brain regions. The data was compiled in an Excel spreadsheet and statistical analyses including a paired t-test were performed using Excel's Analysis ToolPak.

Results

The drinking experiments performed revealed that while undergoing intermittent access to alcohol, the Het A7NesCre animals actually drank more alcohol on average than their WT counterparts. It was initially hypothesized that more AUTS2 staining would be seen in the WT animals, as they carried both copies of the *AUTS2* gene whereas the Het animals only carried one copy. However, the increased alcohol consumption by Hets led to the emergence of another hypothesis--that the Het animals may show higher *AUTS2* expression and dopaminylated histone levels than the WT due to increases in their alcohol consumption.

At first, AUTS2 staining was present in the WT brains, but many of the stains were faint and contained background staining that made interpreting and analyzing the images difficult. Antigen retrieval proved a useful technique in enhancing many of the staining protocols used in this study and producing images appropriate for analysis. This technique was first tested using the same IHC protocol used for AUTS2 and dopaminyl antibodies, but instead used the NeuN

antibody, which allowed for visualization of many cells throughout the mouse brain. Qualitatively, clear differences were seen between the images of NeuN stains that had undergone antigen retrieval compared with those that had not. Images taken from slices that underwent the antigen retrieval protocol showed more clear puncta where labeling was expected, less background staining, and overall clearer images than slices that did not undergo the antigen retrieval protocol. This was first validated through experiments using NeuN to stain neuronal nuclei, which showed diffuse staining throughout the mouse brain (Figure 1). Figure 1 shows the differences in NeuN staining throughout the mouse brain between the two groups.

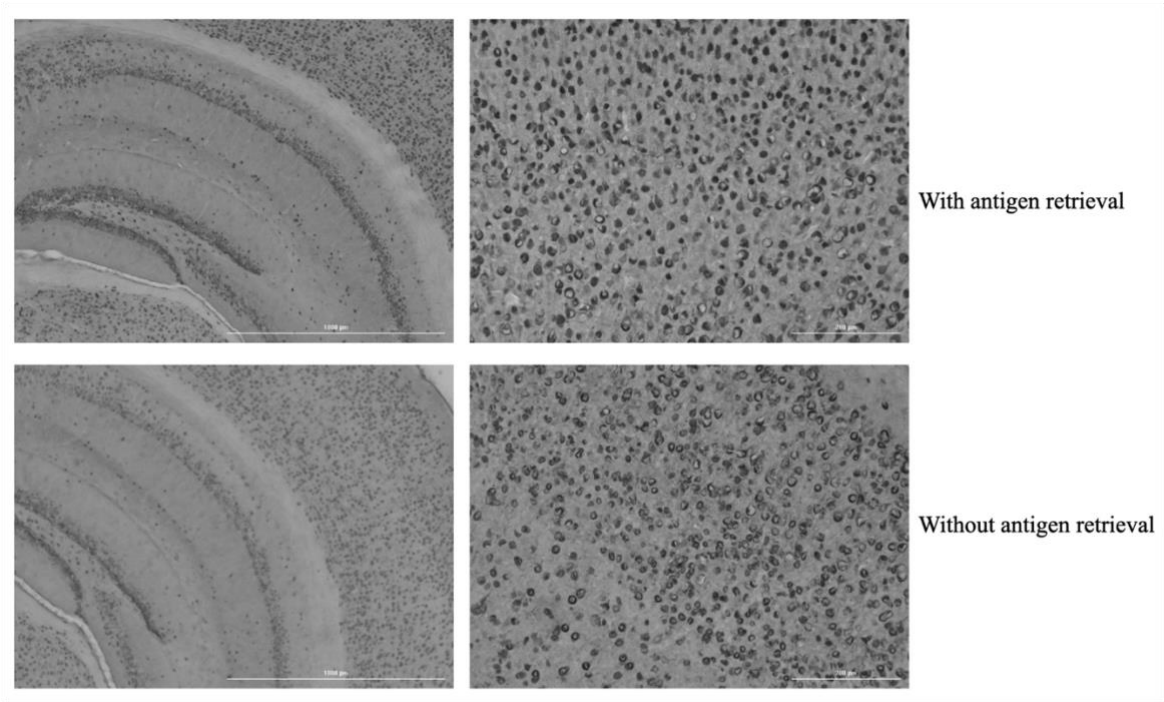

Figure 1. Representative images of the mouse hippocampus (left) at 4x magnification and cortex (right) at 10x magnification, stained with NeuN with and without the addition of the antigen retrieval protocol.

After implementing this technique in experiments involving AUTS2 and histone dopaminylation, crisper images with less background staining were obtained from the AUTS2 IHC staining. However, despite adding the antigen retrieval step, only AUTS2 showed improvements in IHC stain quality and visualization of histone dopaminylation continued to prove difficult. Because of this, another optimization strategy was implemented—adjusting the concentration of the dopaminyl antibody. Different concentrations for anti-H3K4me3Q5dopaminyl were tested and comparisons were drawn between the initial concentration of 1:500 and an adjusted concentration of 1:100. However, this was also ultimately unsuccessful in enhancing the staining. It was determined qualitatively that this adjustment did not lead to any concrete improvements in the stains, though minor changes could be noted in the stain intensity (Figure 2A). Despite this, it was still difficult to visualize any dopaminylated histones in the nucleus accumbens, where we expected to see accumulation (Figure 2A). Some staining was visualized in the caudate-putamen region, but this was determined to be artifactual. Because immunohistochemical methods for dopaminyl staining were yielding few results, immunofluorescence was also used to look for the presence of dopaminylated histones in the regions of interest. Interesting observations were made from the IF images in the cortex and hippocampus of A7NesCre WT and Het animals, but it was difficult to identify dopaminylated histones from the images (Figure 2B). We were unable to prove that these observations did indeed represent dopaminylated histones and thus any data from anti-H3K4me3Q5dopaminyl was unfortunately unable to be validated for this study.

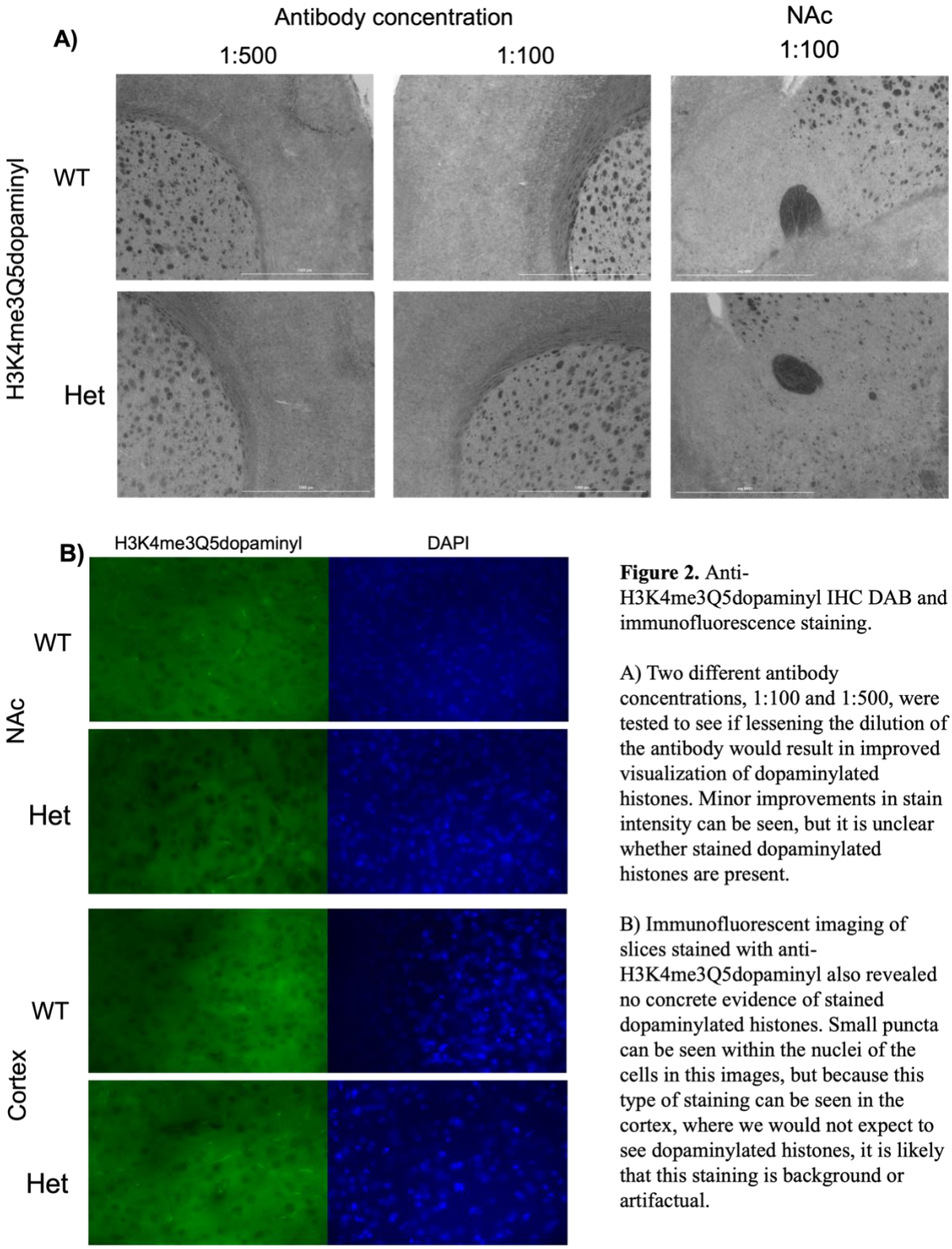

Figure 2. Anti-H3K4me3Q5dopaminyl IHC DAB and immunofluorescence staining.

A) Two different antibody concentrations, 1:100 and 1:500, were tested to see if lessening the dilution of the antibody would result in improved visualization of dopaminylated histones. Minor improvements in stain intensity can be seen, but it is unclear whether stained dopaminylated histones are present.

B) Immunofluorescent imaging of slices stained with anti-H3K4me3Q5dopaminyl also revealed no concrete evidence of stained dopaminylated histones. Small puncta can be seen within the nuclei of the cells in this images, but because this type of staining can be seen in the cortex, where we would not expect to see dopaminylated histones, it is likely that this staining is background or artifactual.

AUTS2 IHC, on the other hand, produced several viable experiments which served to fulfill the questions asked in this study. The results of these experiments are summarized in Figure 3. Firstly, WT AUTS2 animals showed diffuse AUTS2 staining throughout the cortex (both anteriorly and posteriorly), hippocampus, and nucleus accumbens, which was quantified using ImageJ and can be seen in representative images from Figure 3B.

In comparison to the Het AUTS2 animals, AUTS2 positive cells were increased in WT animals only in the anterior cortex ($m_{WT} = 52.6$, $m_{Het} = 39.9$, $t(7) = 2.28$, $p = 0.028$). In the posterior cortex and nucleus accumbens, the Het animals had more AUTS2 positive cells than their WT counterparts (Figure 3A). The mean number of positive cells in the posterior cortex in Hets was 68.9, while in the WT animals the mean was 34.9, only a little over half the number of positive cells in Hets ($t(7) = -5.60$, $p = 0.0004$). In the nucleus accumbens, Hets had a mean of 36.3 positive AUTS2 cells, while WT had a mean of 20.6 AUTS2 positive cells ($t(7) = -3.75$, $p = 0.0036$). No significant differences were observed between WT and Het animals in the hippocampus (Figure 3A).

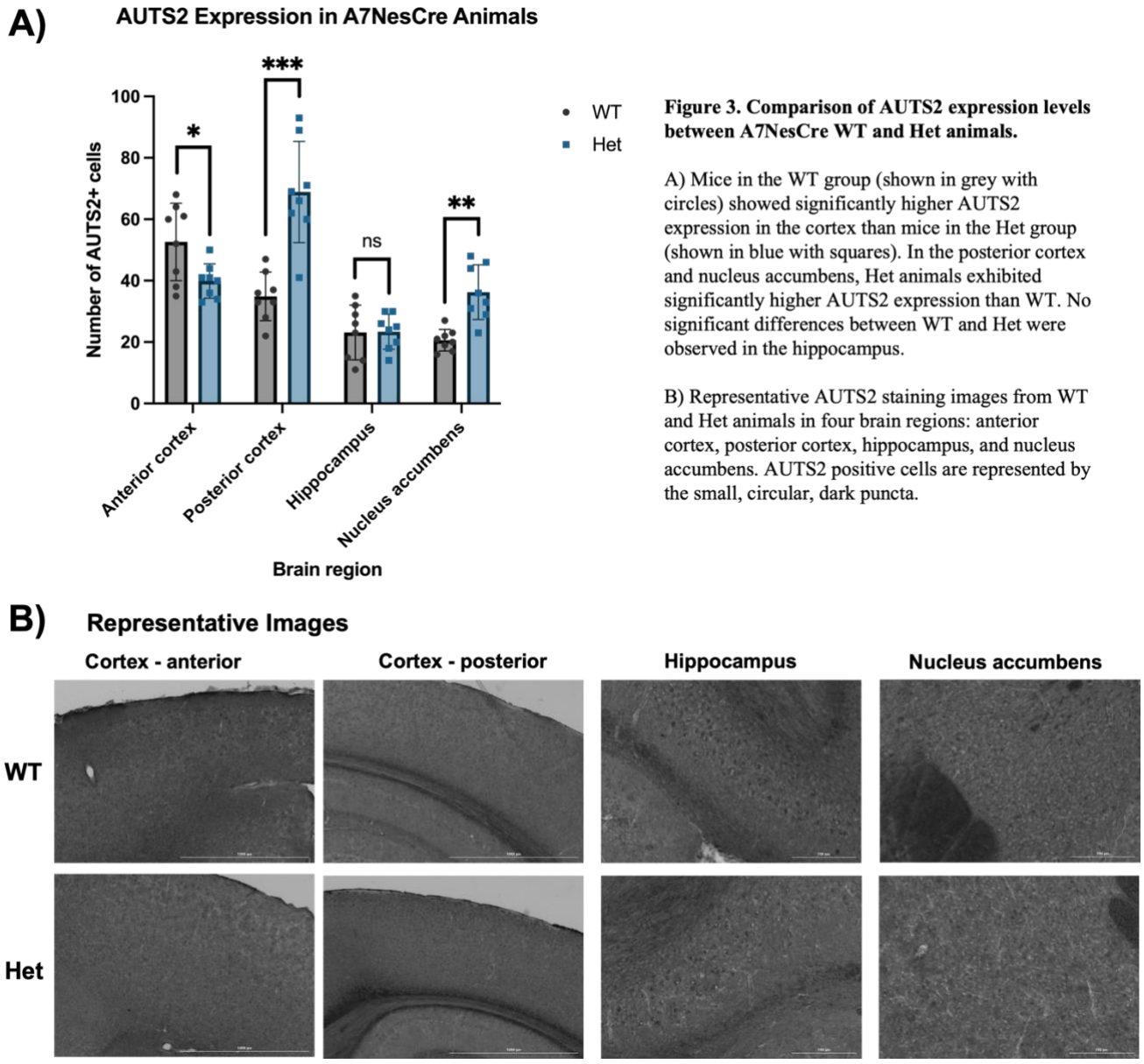

Figure 3. Comparison of AUTS2 expression levels between A7NesCre WT and Het animals.

A) Mice in the WT group (shown in grey with circles) showed significantly higher AUTS2 expression in the cortex than mice in the Het group (shown in blue with squares). In the posterior cortex and nucleus accumbens, Het animals exhibited significantly higher AUTS2 expression than WT. No significant differences between WT and Het were observed in the hippocampus.

B) Representative AUTS2 staining images from WT and Het animals in four brain regions: anterior cortex, posterior cortex, hippocampus, and nucleus accumbens. AUTS2 positive cells are represented by the small, circular, dark puncta.

Limited data was obtained from the animals in the HAP cohort, as the slices from these brains were a little over 2 years old and there was some damage to the tissue. Despite the tissue damage, significant differences were observed between the two animals investigated in the HAP cohort. The number of AUTS2 positive cells was increased in the animal that consumed EtOH compared to the animal that consumed H2O in the anterior and posterior cerebral cortices (dorsal

to the nucleus accumbens and hippocampus, respectively) (Figure 4A). In the anterior cortex, m_{EtOH} = 35.8 and m_{H2O} = 29.8 ($t(3) = 2.55$, $p = 0.042$), and in the posterior cortex, m_{EtOH} = 42.5 and m_{H2O} = 27 ($t(3) = 3.9$, $p = 0.01$). No significant differences between the two groups were observed in the nucleus accumbens, though it can be noted that the mean in the EtOH group was slightly higher, with m_{EtOH} = 29.5 and m_{H2O} = 25.3, though this finding was nonsignificant ($t(3) = 1.44$, $p = 0.12$). Figure 4B shows representative images from the three regions discussed above. Unfortunately, due to tissue damage in the region, data was not acquired for the hippocampal region.

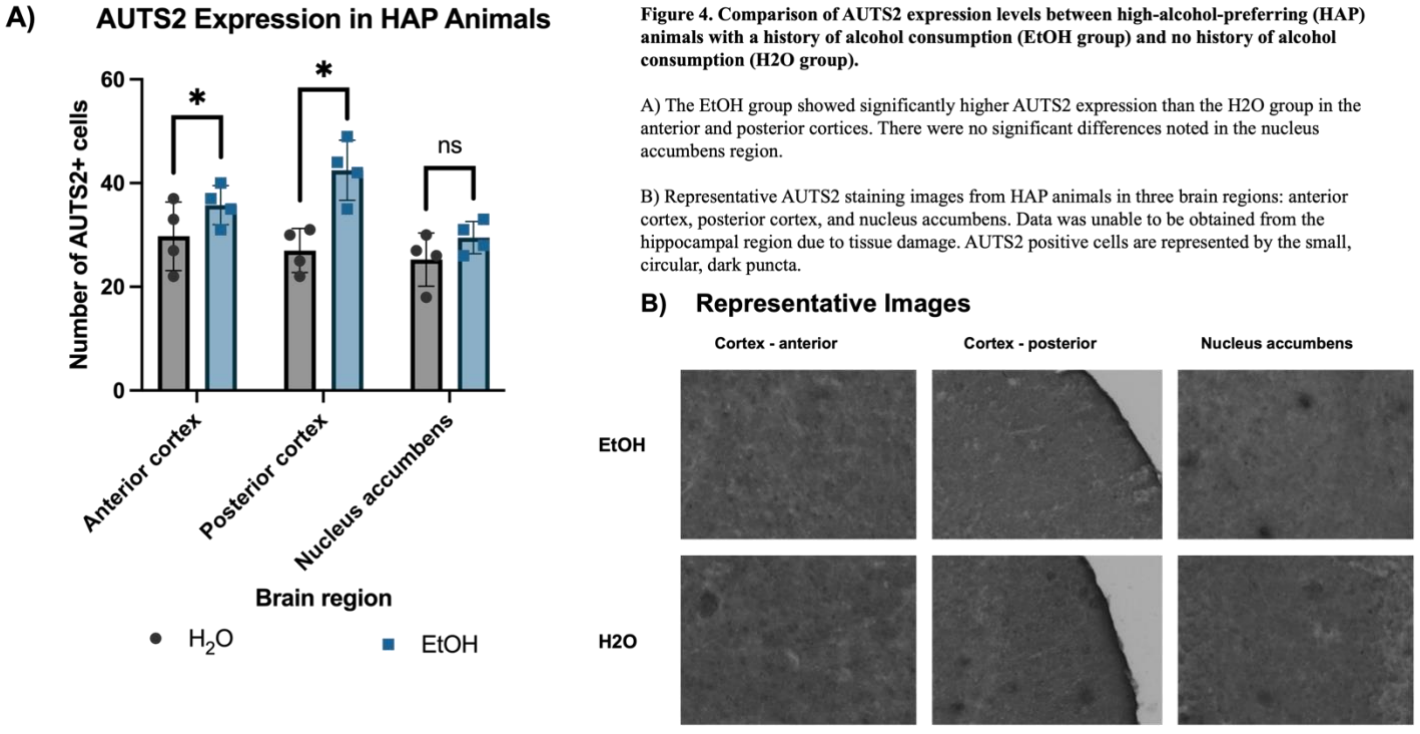

Figure 4. Comparison of AUTS2 expression levels between high-alcohol-preferring (HAP) animals with a history of alcohol consumption (EtOH group) and no history of alcohol consumption (H2O group).

A) The EtOH group showed significantly higher AUTS2 expression than the H2O group in the anterior and posterior cortices. There were no significant differences noted in the nucleus accumbens region.

B) Representative AUTS2 staining images from HAP animals in three brain regions: anterior cortex, posterior cortex, and nucleus accumbens. Data was unable to be obtained from the hippocampal region due to tissue damage. AUTS2 positive cells are represented by the small, circular, dark puncta.

Discussion

In regards to histone dopaminylation, there are factors that could have contributed to the lack of staining seen in the brain slices. Most notably, the antibody used in the study, anti-H3K4me3Q5dopaminyl, has been developed and tested for Western blotting, dot blot, and peptide inhibition assays, but has not been previously used in IHC studies. This could mean that the sensitivity of the antibody is more appropriate for use in studies not requiring IHC. Additionally, a number of studies have been conducted that investigate the H3Q5dop modification in other drug modeling systems, such as cocaine (Lepack et al., 2020) and heroin (Fulton et al., 2022), but little research is available on this modification in alcohol drinking paradigms. In both heroin and cocaine studies, accumulation of H3Q5dop was found and determined to play a role in drug-seeking behavior and abstinence, likely through alterations in gene expression. It cannot be said for certain whether alcohol consumption would have the same effects, though based on what we know about the neural correlates of addiction, it is likely that if a similar study was performed using alcohol as the addictive drug of interest, histone dopaminylation would be seen in the same context. This provides a promising direction for future research in the lab and any future experiments with this antibody should most likely be performed using western blot as the primary technique for revealing accumulation of dopaminylated histones. Another potential direction could be found in the development of an antibody for detecting H3Q5dop with appropriate sensitivity for immunohistochemical applications.

Although IHC along with various optimization techniques were unsuccessful in revealing histone dopaminylation changes in the adult mouse brain, significant data was acquired to demonstrate potential changes in AUTS2 expression in response to alcohol consumption. The

results revealed increased AUTS2 expression in the nucleus accumbens and posterior cortex in the A7NesCre heterozygous individuals compared to WT. However, no significant changes were observed in the hippocampal region, and in the anterior cortex, WT animals showed increased AUTS2 expression compared to Het counterparts. It was initially hypothesized that WT A7NesCre animals would exhibit more AUTS2 staining diffusely throughout the adult mouse brain, as WT animals have both copies of the AUTS2 gene. In Het animals, one copy of the AUTS2 gene was knocked out using Cre recombinase drivers, which theoretically would mean less AUTS2 expression in the brain. Some of the findings of this study contrast with this logic, with a higher number of AUTS2 positive cells found in certain brain regions of the Het animals. As mentioned previously, this finding could be attributed to the fact that the heterozygous A7NesCre animals drank more alcohol than their WT littermates. Because increased AUTS2 expression could be associated with alcohol consumption, it is possible that the increased expression seen in the nucleus accumbens and posterior cortex of Het animals can be attributed to their increased alcohol consumption. The increased expression seen in the nucleus accumbens is a particularly interesting finding because of the established role of this region in the reward pathway, which is activated in response to drugs of addiction, including alcohol. The NAc is one of the central structures involved in the experience of reward and is the site of dopamine release in response to drug intake (Ma and Zhu, 2014). The AUTS2 expression found in Het animals could indicate that their increased alcohol intake correlates with higher AUTS2 expression in a brain region heavily involved in the experience of reward.

However, it is difficult to draw conclusions from these findings, as other regions of the brain, such as the anterior cerebral cortex, showed higher AUTS2 expression levels in WT animals. Data and observations from this region are more consistent with the first hypothesis,

which stated that WT animals will demonstrate more AUTS2 expression due to their possession of both copies of the gene.

Results from the HAP cohort's AUTS2 IHC also show a potential association between alcohol drinking and AUTS2 expression, with increased expression found in the anterior and posterior cerebral cortices of the adult brain of animals with a history of alcohol consumption. Interestingly, there was no difference in expression seen in the NAc between alcohol and water-only consuming mice, which conflicts with the interpretation of the results of the experiments performed in the A7NesCre cohort. Despite this, a slight increase in AUTS2 positive cells was observed in the NAc in Het animals compared to WT and significant differences between the two groups were found in the two other regions investigated. It is important to interpret these results with caution, as slices from only one animal from each group were obtained. This is due to tissue damage in the slices most likely due to their age, which prevented collection of larger quantities of data.

Potential limitations of this study include small sample sizes for both the A7NesCre AUTS2 KO cohort (n = 8) and the HAP cohort (n=2). Larger sample sizes could ensure that the data accurately represent the phenomena demonstrated in this study. Additionally, the increased consumption exhibited by the A7NesCre Het animals was a confounding variable, and through it led to some interesting results, it was a confounding variable nonetheless. Because Hets drank more than WT, it is difficult to determine whether the observed differences in AUTS2 consumption are due to changes in the genetic profile of the animals or if their consumption of alcohol influenced the results. Further studies with an experimental design which allows for clearer comparison between groups could greatly benefit the existing research on AUTS2 and might shed light on the results of this study.

Conclusions

This study helped provide valuable insights into how AUTS2 expression changes in response to alcohol consumption. Data collected from the experiments in the study revealed differential AUTS2 expression levels between wildtype and heterozygous AUTS2 knockout animals as well as between HAP animals that drank alcohol and those that did not. The results showed increased AUTS2 expression in animals that drank more when compared with animals that did not drink or drank less, though this finding was only validated in certain regions of the brain. While this data is somewhat contradictory, it provides a framework for future experiments on this topic and demonstrates potential evidence that alcohol consumption and AUTS2 expression are linked in adult mice. Further understanding AUTS2 and investigating how its expression changes in different alcohol models will help to elucidate its role in AUD and guide future research into this topic. Finding new roles of AUTS2 in association with AUD and the genetic changes that underlie the disorder can help to identify novel predictors and therapeutic strategies for those who suffer from AUD.

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