PROTEOMIC COMPARISON OF TASTE-AVERSION-PRONE AND TASTE-AVERSION-RESISTANT RAT BRAIN REGIONS

by

HEATHER MOSLEY

(Under the Direction of Arthur Grider)

ABSTRACT

Taste Aversion (TA) is a conditioned learning response involving an association between a taste and malaise, resulting in aversion to the taste. TA may be applied to the study of alcoholism. The TA-prone (TAP) and TA-resistant (TAR) rat strains were selectively bred based on TA acquisition. In this study, two-dimensional gel electrophoresis was utilized to analyze the differences in protein expression in the hippocampus, amygdala, and ventral striatum of TAP and TAR rats. M2 pyruvate kinase and myelin basic protein were found at lower levels in TAR rats compared to their TAP controls. Annexin A6, cytokeratin 8, dynamin I, glial fibrillary acidic protein-δ, and sirtuin 2 were found at higher levels in TAR rats compared to TAP rats. Several of these proteins are affected by oxidation, or are involved in metabolism, calcium homeostasis, or cytoskeletal structures and functions. These findings provide useful focal points for future research into TA.

INDEX WORDS: Taste Aversion, Taste Aversion Prone, Taste Aversion Resistant, Alcohol, Oxidation, Proteomics, Hippocampus, Amygdala, Ventral Striatum, 2DE

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CHAPTER 1
LITERATURE SURVEY

Taste Aversion

Taste aversion (TA) is a conditioned response that occurs in the brain when a taste, especially a novel taste, is paired with an aversive stimulus. The taste serves as the conditioned stimulus, while the aversion serves as the unconditioned stimulus. Gastrointestinal distress, such as nausea or vomiting, appears to be the most effective unconditioned stimulus for formation of TA (Garcia et al., 1955; Orr et al., 2001). Conditioned taste aversion (CTA) is observed in many different animals, including mammals, birds, and reptiles (Garcia et al., 1985; Elkins et al., 1991; Bures et al., 1998; Paradis and Cabanac, 2004). While some flavor avoidance or learned preferences have been observed in invertebrates (Bures et al., 1998) these types of behaviors may not be true TA; Paradis and Cabanac (2004) hypothesized that the lack of CTA development they observed in amphibians coupled with a critical review of the work done on invertebrates may imply that only amniotes are capable of developing CTA. This may be due to the brain structures involved in the neural processing of stimuli during CTA learning (Bures et al., 1998; Orr et al., 2001; Paradis and Cabanac, 2004; Yamamoto, 2007). The beneficial implications of CTA are fairly straight-forward: if a food or beverage initiates nausea then avoiding it in the future would be a good way to avoid possible poisoning or pain. Such a learning strategy

would have undoubtedly bestowed a selective advantage on the individuals who employed it, leading to its propagation and current widespread distribution among amniotic animals.

Orr et al. (2001) compiled a list of characteristics of CTA learning that distinguish it from other types of learning. Notable among these are the facts that CTAs can occur after only a single pairing of conditioned and unconditioned stimuli, and the gap between them can last up to several hours without a decrease in the strength of the aversion formed. A delay may in fact be necessary to form very strong CTA; a 10-60 minute interval appears to produce the strongest aversions, while pairings that are closer together or simultaneous produce much weaker CTA (Bures et al., 1998). It is also worth pointing out that CTA is not the same phenomenon as environmental avoidance; CTA is formed when a relationship between taste and illness/nausea is learned; environmental avoidance is not based on nausea, but relies on immediate physical pain (Orr et al 2001, Garcia et al., 1985). Garcia points out that the “skin defense” system that responds to external pain is acting in concert with the “gut defense” system that responds to internal pain, however these two systems can not overlap, so an internal pain cue can lead to the development of gustatory behaviors such as CTA, while an external pain cue could not.

Taste aversion and alcohol

Many different agents can be used as the unconditioned stimulus in TA conditioning, including, but not limited, to: ethanol, lithium chloride (LiCl), cyclophosphamide, drugs of abuse such as cocaine and nicotine, radiation, and physical rotation (Kumar et al., 1983; Garcia et al., 1985; Elkins and Walters, 1990; Elkins, 1991; Bures et al., 1998;). While most of the agents used in CTA have mainly aversive affects, ethanol and other drugs of abuse are notable for having both rewarding and aversive effects. Liu et. al. (2009) studied the formation of CTA in rats with experimentally placed lesions of the gustatory thalamus. Lesions of this area of the brain reduce the efficacy of CTAs formed using rewarding unconditioned stimuli (drugs of abuse). At low doses of ethanol, rats with gustatory thalamus lesions failed to develop, while those rats who received sham operations successfully formed, CTAs. However, at high doses of ethanol there was no difference in CTA formation between the lesioned and sham-operated rats. In this case, the rewarding effects of low doses of ethanol led to a reduction in CTA acquisition because of the gustatory thalamus lesions. Other studies have also demonstrated the differences in effect of low doses of ethanol versus higher doses. Arias et. al. (2009) tested CTA acquisition in weanling Sprague-Dawley rat pups and found that those pups with higher baselines of locomotor activity failed to develop CTAs at the same level of ethanol intake as pups with low baselines of activity. However, both groups of pups developed CTA at the same dosage of LiCl. The difference in TA

conditionability was based solely on the response to ethanol. Age, gender, and social interactions can all play a role in determining the amount of alcohol that an individual will consume. A similar study of adolescent and adult Sprague-Dawley rats showed that adolescents consume higher levels of alcohol than adults. Interestingly, using ethanol as the unconditioned stimulus for TA conditioning, adolescents also require a higher dose than adults to develop CTA; in a social setting (with a cage-mate present) male adolescent rats failed to develop CTA at unconditioned stimulus levels that were normally sufficient. And at lower doses of ethanol, social females actually consumed more of the conditioned stimulus after conditioning (Vetter-O'Hagen et al., 2009). Clearly, younger animals, as well as those in a social situation, responded more vigorously to the rewarding aspects of alcohol than to its aversive qualities.

The balance between the aversive and rewarding aspects of alcohol consumption is one of the keys to understanding the development of alcoholism. The current theories as to the development of alcoholism concentrate on alcohol's effects on disinhibition, reward, habit formation, stress, and inflammation (Zahr et al., 2008).

Many different strains of laboratory animals have been bred specifically to study alcohol intake and effects, including a number of rats and mice. The selection and separation criteria for these strains may be phenotypic, as in the alcohol preferring (P) and non-preferring (NP) rats, or gene-specific, as is the case for knock-out mice (Green and Grahame, 2008; Zahr et al., 2008).

Alcohol Effects

Ethanol oxidation can lead to an increase in the generation of reactive oxygen species (ROS), and free radicals. Free radical damage in response to ethanol has been documented for both the liver and the brain (Review: Albano, 2006). The brain is susceptible to oxidative stress from free radical generation due to the high levels of lipid present in the brain; lipid peroxidation is a measure of oxidative stress.

Alcohol is metabolized in the brain of rats by at least three enzymes: alcohol dehydrogenase (ADH), which plays a minor role, catalase, and Cytochrome P450 2E1 (CYP2E1) (Zimatkin et al., 2006). CYP2E1 is an ethanol-induced enzyme system, which generates a large number of ROS. CYP2E1 was measured at significantly higher levels in the brains of rats that had consumed alcohol than control rats with no alcohol exposure. Exposure of brain homogenates to ethanol also resulted in an increase in levels of CYP2E1 (Montoliu et al., 1994). The increased levels of CYP2E1 were shown to play a crucial role in ethanol oxidation, which in turn led to the formation of free radicals, ROS, and lipid peroxidation in rat brains (Montoliu et al., 1994, 1995).

Alcohol and Oxidative Stress

The metabolic breakdown of ethanol in the brain, which utilizes the three enzymes ADH, catalase, and CYP2E1, produces acetaldehyde. Acetaldehyde is the first metabolite of ethanol, and it increases ROS production in neurons through activating oxidative enzyme pathways as well as increasing the synthesis of nitric oxide (Haorah et al., 2008). One of the three known ethanol

oxidizing enzymes in the brain, CYP2E1, is induced by exposure to ethanol. This may prove to be crucial to understanding oxidative stress caused by ethanol consumption, as CYP2E1 is responsible for high levels of ROS production (Montoliu et al., 1994, 1995; Haorah et al., 2008). Oxidation of ethanol by CYP2E1 in rat brain homogenates causes free radical formation and results in lipid peroxidation (Montoliu et al., 1994). Increased oxidative damage from free radicals and ROS can wreak havoc in cells, resulting in neuronal cell death (Haorah et al., 2008). Ethanol-induced CYP2E1 is of particular interest due to increased levels of this enzyme in response to ethanol exposure (Montoliu et al., 1994,1995; Upadhya et al., 2000), which could lead to high levels of this enzyme in individuals with chronic exposure to ethanol, such as alcoholics.

Antioxidant Proteins in the Brain

The brain has multiple systems in place to protect itself from oxidative damage (Montoliu et al., 1994, 1995; Yu, 1994; Schafer and Buettner, 2001). Enzymes such as superoxide dismutase (SOD) are crucial in protection from free-radical generated oxidative damage. SOD metabolizes the radical superoxide (O_2•) to form H_2O_2 , thereby reducing oxidative stress (Yu 1994). SOD was shown to reduce ethanol-induced lipid peroxidation in rat brains (Montoliu et al., 1994).

Glutathione (GSH) is a tripeptide which reacts with ROS to reduce cellular oxidative stress. It consists of one cysteine and two glutamate residues. The sulfur moiety of the cysteine reacts with ROS, thereby forming oxidized glutathione dimers (GSSG). Oxidation subsequently causes the depletion of

cellular GSH levels (Schafer and Buettner, 2001), leaving the cell susceptible to oxidative damage if ROS continue to be generated. GSH levels were significantly decreased in rat astrocytes in response to ethanol exposure (Montoliu et al., 1995). The presence of higher levels of proteins such as GSH or SOD in tissues could provide protection from oxidative stress such as that produced by ethanol metabolism. Higher constitutive levels of antioxidant proteins would be depleted more slowly, and individuals with these levels could withstand oxidative stress with less damage than individuals with lower levels.

Alcohol and the Brain Reward System

The hippocampus, the amygdala, and the ventral striatum are part of the mesocorticolimbic system, and play a crucial role in reward circuitry as well as in learning of conditioned behaviors (Everitt et al., 1999; Bowirrat and Oscar-Berman 2005; Robbins et al., 2008).

MRI studies have shown decreased volume of the reward pathway, specifically in the amygdala and the nucleus accumbens, a structure that is contained within the ventral striatum, in chronic alcoholics, even after periods of abstinence (Everitt et al., 1999; Heimer and Van Hoesen 2006; Makris et al., 2008). Proteomics studies of certain strains of rats selectively bred for alcohol preference, such as the P/NP strain, have shown differential protein expression in response to ethanol exposure between strains (McBride et al., 2009). In these studies, both the amygdala and the nucleus accumbens shell within the ventral striatum expressed several proteins differently between ethanol naïve and ethanol exposed individuals (Bell et. al., 2006).

Yamamoto (2007) hypothesized that the amygdala, and specifically the central nucleus and the basolateral nucleus of the amygdala, play a role in the development of CTA. Lesions were induced in the central nucleus and the basolateral complex of the amygdala of rats to test the role of the amygdala in TA conditioning to novel and familiar tastes. Rats were exposed to 4 conditioning sessions, prior to the test day. Rats with lesions of the basolateral complex took longer to form CTA to novel tastes than controls. However, lesions of the central nucleus had no effect on TA formation. These data would implicate a role of the basolateral complex of the amygdala in recognizing novel taste stimuli, which would affect CTA formation (St. Andre and Reilly, 2007). Under normal circumstances CTA is acquired more quickly to novel tastes, while familiar tastes suffer from latent inhibition, and will take a longer time and more conditioned and unconditioned stimuli pairings to develop CTA (Bures et al., 1998; Orr et al., 2001). After exposure to TA conditioning, the neurons of the basolateral amygdala have increased excitability; and taste-reactive cells in this area have a greater rate of suppressed excitability when exposed to the conditioned stimulus (Kim et al., 2010). The central nucleus of the amygdala may also play a role in regulating alcohol consumption (McBride, 2002). Hill et al. (2001) studied adolescents at high risk of developing alcoholism (defined as having an average of four alcoholic first or second degree relatives) and found that the right amygdala of these individuals was smaller than in low-risk control subjects. These structural differences were present even without drinking alcohol.

The role of the amygdala in TA learning, as well as its affects on alcohol drinking behavior and its effects from alcohol exposure all indicate that this area of the brain may have functional differences that would be emphasized in selection for TA performance, as in the case of TA prone (TAP) and TA resistant (TAR) rat strains, which will be discussed further in the preliminary data section.

The hippocampus also appears to play a role in certain aspects of TA learning, as well as being affected by alcohol consumption. In an MRI comparison-study of adolescent-onset alcohol use disorders, those individuals who had abused alcohol during their adolescence had lower hippocampal volumes than their age-, sex-, and handedness-matched controls. However, there was no difference found in total cerebral volume or amygdala volume in these cases (DeBellis et al., 2000). These finding are only corollary in nature, and do not demonstrate why the hippocampal volume is decreased in those individuals with adolescent-onset alcohol abuse.

Studies such as these would indicate that ethanol metabolism may have a more profound impact on the regions of the brain involved in reward, particularly with longer periods of chronic exposure. However, it is also possible that there are volume deficits, or other physiological differences, in these brain areas prior to ethanol exposure that might predispose individuals to higher levels of consumption such as those seen in alcoholism. While it is possible that the consumption of high levels of alcohol may lead to the reduced volume, it is also feasible that the difference predates alcohol consumption, and may be a risk factor for the development of alcohol use disorder. However, a separate MRI

study comparing volume in children and adolescents at risk of alcoholism (based on family history) showed no difference in hippocampal volume (Hill et al., 2001).

Analysis of brain tissue using fluorescence in situ hybridization found high constitutive levels of CYP2E mRNA in neurons of the hippocampus in both rat and human brain (Uphadya et al., 2000), giving further credence to the idea that this area is at higher risk of oxidative damage from alcohol consumption.

Proteomics studies of human alcoholic hippocampus tissues have also illustrated the differential protein expression brought about by chronic alcohol consumption. Proteins involved in energy and protein metabolism, signaling, vesicle transport, cytoskeleton, and oxidative stress were all altered in tissues from alcoholics compared to non-alcoholics (Matsuda-Matsumoto et al., 2000).

Lesions of the hippocampus (dorsal and ventral) affect long trace conditioning, or CTA acquisition with a long delay between the conditioned and unconditioned stimulus. Rats were offered a novel conditioned stimulus paired with an intra-peritoneal (i.p.) injection of LiCl 3 hours later, and intake of the conditioned stimulus was measured after 2 days. The same rats were offered a new novel conditioned stimulus with an immediate i.p. injection, and conditioned stimulus intake was again measured 2 days later. In these rats, lesions of the hippocampus led to reduced CTA formation for the long delay conditioning, but no difference was seen between rats with lesions and sham-operated rats in the no-delay CTA conditioning (Koh et al., 2009).

As this body of evidence grows, researchers must ask whether it is the effects of alcohol consumption that lead to these neurochemical and

physiological changes, or if these changes predate alcohol exposure, and could indicate increased susceptibility to developing alcoholism. Results such as these indicate that there could be differences in brain chemistry prior to ethanol exposure between lines selectively bred for differential responses to ethanol, or those who are at higher risk of developing alcoholism. This conclusion could also be applied to rat strains selectively bred for response to a different stimulus, such as taste aversion, that exhibit similar preferences for ethanol.

Taste Aversion Applications

CTA use for medical or practical purposes has been studied extensively. While CTA was used to limited success in treating human patients for alcoholism (Elkins, 1991), more recent work had placed greater emphasis on its application in wildlife management (Gustavson et al., 1976, 1985; Rusiniak et al., 1976; Gill et al., 2000). Since CTA is non-lethal, its use is not only effective in those areas where wildlife professionals may wish to keep the animals in question at the location, but also in those areas where humans and their pets or other domesticated animals may come into contact with baits (Maguire et al., 2009).

Early work with predator control applications of CTA focused on canids (Gustavson et al., 1974, 1976, 1985; Ellins and Catalano, 1980; Burns, 1980) with some initial mixed results. The experiments by Burns et al. failed to develop CTA to chickens in coyotes, but this failure may be due to several reasons. Firstly, the coyotes were pre-exposed to the conditioned stimulus through live chicken presentation for three days prior to conditioning. Such exposure would

likely result in latent inhibition, thus requiring multiple conditioned and unconditioned stimulus pairings prior to CTA acquisition (Bures et al., 1998; Orr et al., 2001). The unconditioned stimulus doses of LiCl were liquid, and were injected centrally during initial trials and later brushed over the carcasses, allowing the coyotes to taste the salty LiCl (Burns, 1980). This may have led to learned aversion of the salty taste, rather than CTA of the prey item. However, other studies produced positive results, and were able to develop CTAs in captive (Gustavson et al., 1974) and wild (Ellins and Catalano, 1980) coyotes. The keys to effective CTA from these studies would seem to be that the aversive agent is undetectable, and that there are multiple pairings offered. Gustavson et al. (1974) hypothesizes that the initial CTA developed to the taste of the prey is not sufficient to overcome the predatory behavior in response to the prey animal, but further conditioned and unconditioned stimuli pairings can eventually lead to complete CTA of the prey animals themselves, so that even the sight or smell of the prey would lead to avoidance behavior.

Humans and rats both acquire TA quickly when presented with new tastes, though it can also be applied to tastes that have been present without ill effects in the past (Bures et al., 1998; Orr et al., 2001). For example, TA conditioning has been used as a treatment for alcoholism with some success (Review: Elkins, 1991). It was noted that there is a genetic component to TA acquisition, where some individuals may acquire TA readily, while others do not (Elkins, 1986). Alcoholism, as well as abuse of other drugs, may have a genetic

component, and it was hypothesized that taste aversion could play a role in the development of alcoholism/drug abuse (Elkins, 1986; Orr et al., 1997, 2004). If differences in brain chemistry could be identified, these differences could help to diagnose those who may have a predilection towards developing abusive behaviors towards drugs, including alcohol. Such information could help those at risk to avoid developing such behaviors. Perhaps an understanding of the underlying physiological reasons for addiction could lead to a pharmacological treatment.

Hypothesis

It is hypothesized that TAR rats exhibited increased antioxidant protein expression compared to TAP rats. To explore this hypothesis, the patterns of protein expression in the amygdala, hippocampus, and ventral striatum of TAR and TAP rats were compared using the proteomic techniques two-dimensional gel electrophoresis and mass spectrometry.

Preliminary Data

It was noted that there is a genetic component to TA acquisition, where some individuals may acquire TA readily, while others do not. This observation led to a project to selectively breed rats for their ability to acquire TA conditioning (Elkins, 1986). TA Prone (TAP) and TA resistant (TAR) rat strains were selectively bred from a Sprague-Dawley derived line over 27 generations. The selection criterion was TA conditioning response, with TAP rats readily developing TA, while TAR rats showed minimal TA. The rats were exposed to a novel saccharin solution (conditioned stimulus), paired with an emetic chemical

injection (cyclophosphamide – unconditioned stimulus). After this initial TA conditioning, the rats were given free access to both the saccharin solution and water in their home cages, with daily measurements of the intake of both liquids (a two-bottle free choice set-up). After three days the average saccharin solution intake was calculated and used to determine each rat's preference score. Using the intake preference, strain assignments were made. Rats that consumed the highest amounts of the conditioned stimulus solution were placed into the TAR line, while those that consumed the least were placed in the TAP line (Elkins, 1986). As discussed earlier, TA and environmental avoidance are two fundamentally different responses, and it was hypothesized that they utilize different pathways in the brain. Shock-motivated environmental avoidance (SMEA) was used as a control to ensure that TA conditionability was being selected for, and that there were not similar effects on other aversive learning behaviors such as environmental avoidance. The strain divergence actually resulted in the opposite response to SMEA as to TA, with TAP rats performing poorly on SMEA trials, while TAR rats' performance increased (Elkins, 1986).

TA proneness was shown to have a substantial genetic component by the separation of the TAP/TAR lines. Ethanol had been shown to be an effective TA aversive stimulus that resulted in development of TA in TAP rats and a lack of TA development in TAR rats (Elkins et al., 1992). Following analysis of these results, the investigators posed the question of whether or not TAP and TAR strains might serve as a model of alcoholism. As alcoholism also has a genetic

component, it was hypothesized that this strain might offer a new way to study the development of alcoholism (Orr et al., 1997, 2004; Zahr and Sullivan, 2008).

After Elkins et. al. (1992) demonstrated the TA producing qualities of ethanol injection in TAP, but not TAR, rats it was hypothesized that the two strains would differ in free-choice consumption of ethanol. If TAR rats failed to develop TA when injected with ethanol, surely they might also choose to consume more ethanol when it was offered.

Rats were given access to both the ethanol solution and water in their home cages using a rotating, two-bottle free-choice system. The position of the bottles was changed daily, and every three days the concentration of ethanol in solution was increased by 1%.This experiment showed conclusively that TAR rats did consume more ethanol than TAP rats at ethanol concentrations from 2-10% (Orr, 1997).

To further study ethanol self-administration, TAP and TAR rats were offered a limited, 2-hr access to 10% ethanol for nine days, followed by an unlimited, 24-hr access to 10% ethanol for nine days. The same rotating two-bottle system described above was used. For both the limited and unlimited access designs, TAR rats drank significantly more ethanol than TAP rats (Figure 1) (Orr et al., 2004)

To rule out confounding variables that might lead to this result, ethanol clearance was calculated by analyzing blood alcohol content (BAC) from tail vein blood collections at 1, 4, and 7 hrs following a 2.5 g/kg body weight i.p. injection of ethanol (Figure 2). There was no difference in clearance between the two

lines. Consummatory behavior of food and water was also measured for a week to rule out higher base levels of consumption in TAR rats; both lines consumed the same amount of food measured in g/kg body weight (Orr et al., 2004).

Preliminary data from this laboratory has shown an increase in levels of SOD activity in the amygdala of TAR rats compared to TAP rats (Figure 3). It is possible that the presence of higher levels of SOD activity would offer a protective mechanism from the production of ROS and free radicals generated during the oxidation of ethanol. The removal of superoxide generated in the presence of ethanol would protect this portion of the brain from oxidative damage that could differentially affect TAP rats. If TAR individuals were protected from the negative effects of ethanol, then they would be free to exhibit an increased response to the rewarding aspects of it, which could explain their increased self-administration of alcohol.

The current study is designed to determine if there are protein differences between the brains of TAP and TAR rats that could result in protection from oxidation. If so, then future studies will be designed to quantify any associated affects of ethanol consumption among those antioxidant proteins. The information learned from studying such differences in taste aversion pathways will enhance our understanding of mechanisms associated with the risk of developing alcoholism.

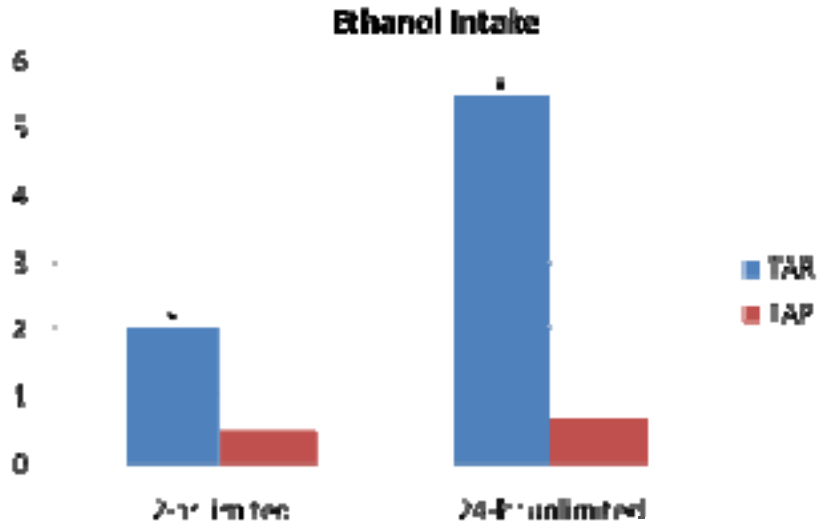


Figure 1: TAP vs. TAR intake of 10% ethanol solution in g/kg body weight. (Orr et al., 2004) *p≤0.05

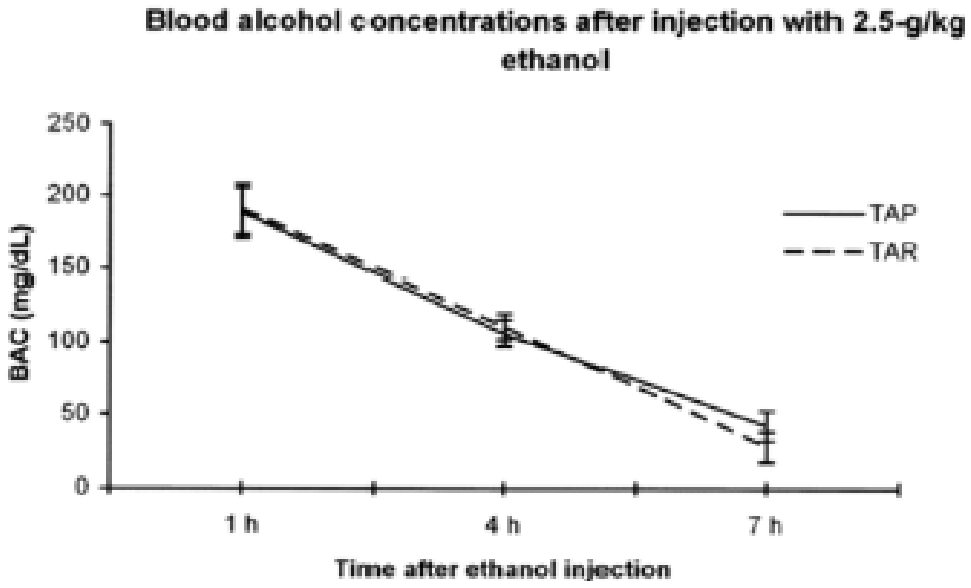


Figure 2: TAP and TAR blood alcohol concentrations. This graph plots the clearance of ethanol from the blood of TAP and TAR rats after a 2.5 g/kg body-weight injection. Blood alcohol content (BAC) was measured 1, 4, and 7 hours after the injection at 0 hours. (Orr et. al., 2004)

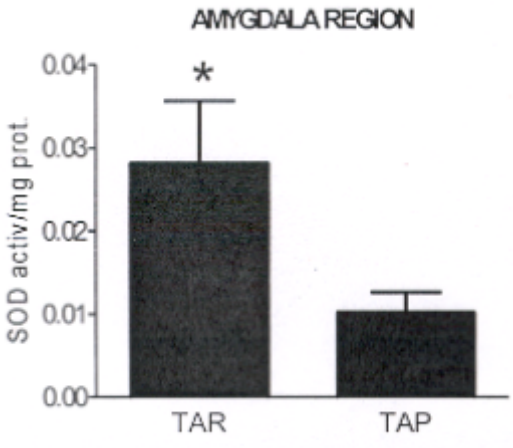


Figure 3: Preliminary data showing increased superoxide dismutase activity in the amygdala of TAR rats compared to TAP rats. *p<0.05

CHAPTER 2

EXPERIMENTAL

Tissue Samples

The animals to be used in this experiment were bred, housed, and cared for at the Augusta State University vivarium in Augusta, Georgia under approval of the Augusta State University Animal Care Committee. All subjects were ethanol-naïve two-year old adult rats of the Sprague-Dawley derived TAP and TAR strains (detailed in literature survey - preliminary data). Following protocols approved by the University of Georgia IACUC (Institutional Animal Care and Use Committee) the rats were euthanized by decapitation and the brains were quickly removed and stored at -80°C until dissected. Four brains were available for this experiment, all from two year old individuals, both TAP samples from female rats; TAR samples from one male and one female individual. The hippocampus, amygdala, and ventral striatum were dissected by hand using a #11 scalpel blade. Dissection was performed in a cryostat kept at -24°C. The hippocampus was dissected using the visual differentiation between it and the surrounding tissue. The amygdala was dissected using the cerebral peduncle on the ventral surface of the brain as the lateral boundary, the optic chiasm as the rostral boundary, and the mammilary bodies as the caudal boundary. The ventral striatum was dissected as a block of tissue approximately 3 mm thick extending approximately 2.5 mm to either side of the midline from the optic chiasm (caudal

border), and extending rostrally approximately 3 mm. Tissues were immediately placed in Eppendorf tubes on dry ice until they were stored at -80°C.

Protein Extraction and Measurement

All samples were weighed prior to extraction procedures; each tissue type was processed separately. The tissues were pulverized using a metal mortar and pestle (Biospec Products, Inc.) in liquid nitrogen, and immediately transferred to 1.5 ml Eppendorf tubes containing 1 ml BIO-RAD Ready Prep™ Sequential Extraction Kit Reagent 1 (40 mM Tris base) and 10 µl protease inhibitor cocktail (Sigma). The samples were sonicated on ice using a Branson Sonifier® S-450 (four 30 sec blasts, at least 60 sec incubation on ice between each blast). The samples were centrifuged for 10 min at 13,000 rpm at 4°C. The supernatant was collected and stored as the soluble fraction. The pellet was washed twice with a half volume of Reagent 1 (vortexed 1-2 min, centrifuged 10 min at 13,000 rpm at 4°C) before a half volume of Reagent 3 (5M urea, 2M thiourea, 2%w/v CHAPS, 2%w/v SB 3-10, 40mM Tris, 0.2%w/v Bio-Lyte 3/10 ampholyte) was added. This was vortexed for 5 min and sonicated twice with 10 sec pulses on ice to fully re-suspend the pellet, then centrifuged 10 min at 15,000 rpm. The supernatant, which was the insoluble fraction, and the highly insoluble pellet were stored at -80°C.

Both the soluble and insoluble fractions were reduced and alkylated using Sigma ProteoPrep™ Tributylphosphine (TBP) and Alkylating Reagent Iodoacetamide (IAA). Reduction and alkylation was performed prior to the Isoelectric focusing (IEF) step to protect against disulfide bridge formation

between cysteine residues, and to produce better visualization of protein spots on 2-D gels (Herbert et al., 2001). Reduction agent TBP was added at 5 mM and incubated for 1 hr; IAA was added at 15 mM and incubated for 1.5 hr to alkylate.

Protein concentration was calculated using the BIO-RAD RC/DC Protein Assay, utilizing the Lowry high sensitivity assay. Absorbance was measured at 750 nm using Beckman Coulter DU800 spectrophotometer. The concentration was then used to calculate the volume of homogenate needed for 300 µg of protein.

The soluble protein fractions from all three brain regions contained a high enough concentration of protein to provide 300 µg in duplicate. The calculated volume of protein solution was combined with double distilled water (ddH_2O) to reach an even volume of 100, 200, or 300 µl; these volumes were divided into 100 µl aliquots in 1.5 ml Eppendorf tubes for delipidation. Samples were delipidated and precipitated using 1:12:1 TBP: acetone: methanol (TAM) solution. The TAM solution was added to the protein homogenate sample at a concentration of 14:1 (1.4 ml : 100 µl) and incubated on ice for 90 min, after which it was centrifuged for 15 min at a low speed (2800 xg) at 4°C to precipitate the protein. The supernatant was removed and discarded. The protein pellet was then washed sequentially with 0.2 ml each of TBP, acetone, and methanol, centrifuging for 5 min at 10,000 rpm at 4°C for each wash. After removing the final supernatant, the pellets were then air-dried upside-down. The delipidation step was added due to the presence of high levels of lipids in brain tissue; this

procedure should result in higher protein solubility and decreased streaking in the second dimension gel (Mastro and Hall, 1999).

The insoluble protein fractions had lower protein concentration scores than the soluble fractions. The hippocampus and amygdala samples contained enough protein for one complete set of gels at 300 µg of protein, however the ventral striatum samples did not. The calculated volume of protein solution was combined with double distilled water to reach an even volume of 100 or 200 µl per Eppendorf tube. The samples were acetone precipitated overnight at -20°C using 4:1 concentration of acetone. After precipitation, the samples were centrifuged for 30 min at 13,000 rpm at 4°C. The supernatant was discarded, and the pellets were air-dried upside-down.

Two-Dimensional Gel Electophoresis (2DE)

The pelleted sample was combined with 125 µl rehydration buffer (PSB, TBP, ampholyte, bromophenol blue), loaded on immobilized pH gradient strips (7 cm pH 3-10; BioRad) and the strips were rehydrated using active re-hydration overnight in the BIO-RAD Protean IEF cell. The gel strips were covered with mineral oil to prevent dehydration. About 15 hours later, paper wicks wet with 10 µl ddH_2O were placed between the strips and the electrodes, and the IEF run was turned on; the samples received 20,000VH using a rapid ramp setting. Upon completion of IEF, the gels were removed, drained of mineral oil, and stored at -80°C.

The second dimension run used denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fresh 10% polyacrylamide gels

were made and allowed to set. The completed IEF strips were thawed at room temperature for 15 min, and incubated in 2 ml IEF buffer each (6M urea, 0.375 M Tris, 2% SDS, 20% glycerol, TBP) for 20 min on a shaker table. The IPG strips were then placed on top of the SDS-PAGE gels, in Tris-glycine-SDS running buffer (10X TGS, ddH_2O). The gels were run using Hoefer SE600 electrophoresis units, and a Consort EV265 power supply at 30 mA/gel. The runs were stopped when the dye front has migrated approximately 9 cm. The gels were then fixed using 10% acetic acid, 40% methanol solution, washed three times with ddH2O and left to soak overnight in Blue silver stain (10% phosphoric acid, 10% ammonium sulfate, 20% methanol, 0.12% Coomassie G-250) (Candiano et al., 2002, 2004). The blue silver stain was poured off, and fresh ddH_2O was added and replaced as needed to de-stain the gels.

The gels were scanned using HP Precisionscan Pro 3.1 on an HP Scanjet automatic document feeder scanner. After scanning the gels were stored in a refrigerator (4°C). Phoretix™ 2D Advanced v6.01 software (Nonlinear Dynamics Ltd) was used to quantify protein spot concentrations. All spots on the gels were selected and matched manually, with each fraction from each of the brain sections processed individually. After subtracting the background, spots were normalized and compared by volume. Those spots with a 2-fold or higher change between the TAP and TAR treatments were chosen for further analysis. The soluble protein samples ($n \geq 4$ due to multiple runs) were further narrowed down by using a paired Student's t-test, $p \leq 0.05$, to test for statistical significance. Spots of interest were excised by hand from the gels and sent to the Proteomics &

Mass Spectrometry Core Laboratory at the Medical College of Georgia in Augusta, GA for in-gel tryptic digestion, mass spectrometry (MS), and MS database query to identify proteins. The Proteomics & Mass Spectrometry Core Laboratory uses the Mascot, ProFound, and Protein Prospector search engines to search MS databases.

CHAPTER 3

RESULTS

Overview

Duplicate tissue samples were obtained for each genetic group. The tissue samples from all three regions were pre-fractionated into the soluble and insoluble protein fractions prior to 2DE and subsequent MS for identification. In those fractions that were analyzed multiple times, spots missing from two or more gels of the same genotype were excluded from further analysis, unless all of the replicate gels from that genotype did not have the spot.

The amount of protein contained in the soluble protein fractions ranged from 1.15 mg/ml to 16.57 mg/ml, with the hippocampus having the most, and the ventral striatum having the least. Within the insoluble protein fractions, the ventral striatum did not yield utilizable protein levels while the hippocampus had the most (Table 1). The proteins which exhibited at least two-fold differences between genotypes were identified by MS (Table 2). All of the proteins, except the glial fibrillary acidic protein from the insoluble hippocampus fraction, exhibited a confidence index percent score of >90%. The insoluble hippocampus glial fibrillary acidic protein is included since the same spot from two separate gel separations were submitted for analysis and yielded the same identification despite the protein score.

Soluble Fractions

The soluble amygdala fractions were run twice per sample. There were roughly 118 protein spots for this region (Figure 4A and B). We chose one spot from these gels that was decreased in TAR ($p = 0.0068$). The protein was identified as M2 pyruvate kinase (Figure 5). Pyruvate kinase is the final enzyme in glycolysis, which catalyzes the formation of pyruvate from phosphoenol pyruvate, producing ATP in the process. This step is often the rate-limiting step in glycolysis. There are two pyruvate kinase genes, L and M; the M gene produces two isoforms/isoenzymes, M1 and M2 (Hance et al., 1982; Dabrowska et al., 1998; Jurica et al., 1998). M2 is present at the highest concentration in cells that are growing and dividing, such as embryo and fetal tissue, stem cells, and cancer cells (Mazurek et al., 2005, 2010; Christofk et al., 2008 a, b).

The soluble hippocampus fractions were run three times for each sample. There were approximately 119 spots on these gels (Figure 6A and B). We chose four spots from these gels for MS. One returned with a protein score CI of 0, and was excluded from the results. The three others were all increased in TAR, and were identified as dynamin I (two IDs; $p=0.0052$, $p=0.051$) and glial fibrillary acidic protein-δ ($p <0.0001$) (Figure 7). The two protein spots that were identified as dynamin I were located in extremely close proximity to each other on the gels; this small separation may be due to a slight modification or break of the protein in the two spots, or may indicate different isoforms of this protein. Dynamin I is one of three dynamin genes (dynamin I, II, and III) which are expressed in mammalian tissues. There are at least 25 different splice variants between these

three genes, and 21 of them are found in rat brain tissue (Cao et al., 1998). While dynamin II is found ubiquitously throughout the cells of the body, dynamin III is only found in certain tissues and dynamin I is almost exclusively localized to neural tissue (Faire et al., 1992; Sontag et al., 1994; Warnock et al., 1995; Cook et al., 1996; Cao et al., 1998). The other spot was identified as glial fibrillary acidic protein. Glial fibrillary acidic protein-δ is a member of the glial fibrillary acidic protein family of proteins, which contains three other alternate splice-variants: alpha, beta, and gamma (Condorelli et al., 1999; Eng et al., 2000; Perng et al. 2008). Glial fibrillary acidic protein is a component of intermediate filaments in astroglial cells; while some of the other alternative splice variants of glial fibrillary acidic protein are found in the peripheral nervous system or in non-neural tissue, glial fibrillary acidic protein-δ is specific to brain and spinal cord tissue (Condorelli et al., 1999; Perng et al. 2008).

The soluble ventral striatum fractions were run twice per sample. These gels had approximately 94 spots (Figure 8A and B). There was one spot chosen for MS, which was increased in TAR ($p=0.009$). This protein was identified as keratin complex 2, basic, gene 7 or cytokeratin 8 (Figure 9). The cytokeratins are structural proteins that come together to form intermediate filaments, which are found in the cytoskeleton and the nuclear envelope. Intermediate filaments which are composed of cytokeratins require Type I, which are acidic proteins, and Type II fibers, which are neutral or basic (Review: Steinert and Roop, 1988). Cytokeratin 8 is functionally and genetically the same as the Cytokeratin endo A protein isolated in rats and mice (Hashido et al., 1991).

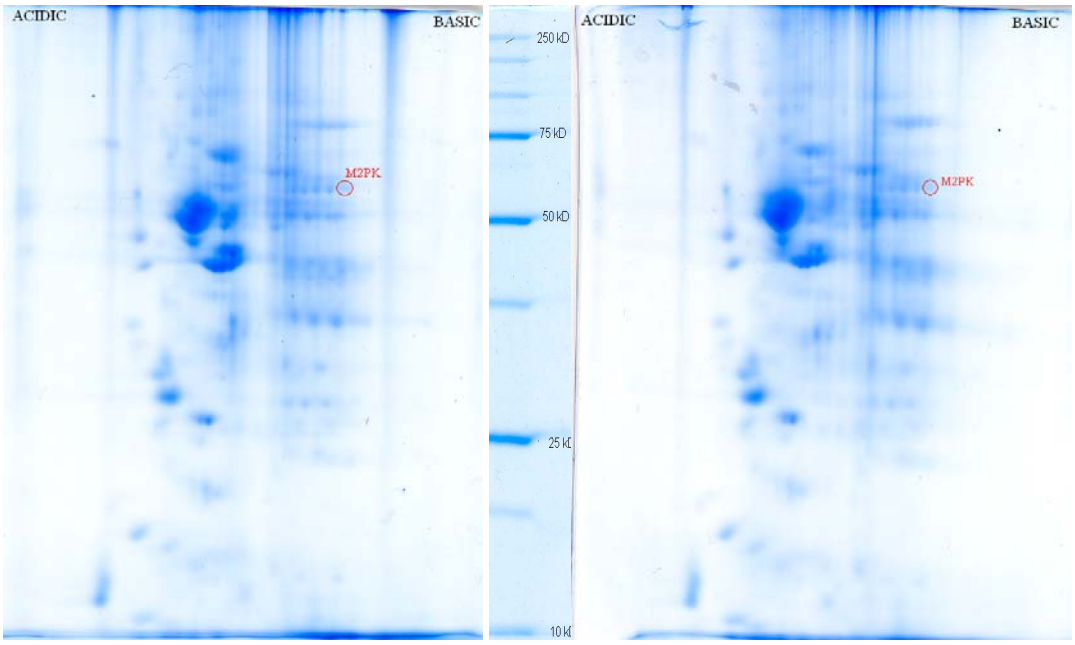
Insoluble Fractions

The insoluble fraction of the hippocampus was the only insoluble region that was analyzed. The insoluble hippocampus fractions were run once per sample (n=2). There were approximately 49 spots on these gels (Figure 10A and B). Only spots that had agreement between both samples of a treatment were included in analysis (either both present or both absent). The n-value was too small to run a t-test. There were 9 spots chosen for MS, and while four of these returned with a protein score confidence interval (CI) of 0 and were excluded from the results, five were identified. Of those, 4 were increased in TAR: annexin A6, sirtuin 2 (two IDs for this protein), and glial fibrillary acidic protein-δ (Figure 11).

Annexin is a protein that binds with phospolipids, particularly those in cell membranes, in a Ca^{2+}-dependent manner. Annexin A6 appears to play a role in membrane vesicle aggregation, endocytosis, and budding of clathrin-coated vesicles (Kamal et al., 1998; Gerke and Moss, 2002). Annexin A6 binding to the plasma membrane appears to play a regulatory role in Ca^{2+} homeostasis within the cell. An increase in the intracellular Ca^{2+} level will promote annexin A6 binding to phospholipids (Gerke and Moss, 2002, Skrahina et al., 2007; Monastyrskaya et al., 2009). The two spots identified as sirtuin 2 were located in close proximity to each other on the 2DE gels, at roughly the same molecular weight but slightly different isoelectric point (pI). Sirtuin 2 is part of a larger group of acetylases known as SIRs (silent information regulators) (Finnin et al., 2001; North et al., 2003). Sirtuin 2 is notable for the presence of an NAD-binding

domain, a zinc-binding domain, and a protein binding site that are class-specific (Finnin et al., 2001). While it was originally thought to act only as a histone deacetylase, sirtuin 2 has been shown to preferentially associate with α-tubulin, and deacetylate it on the Lys-40 residue (North et al., 2003). The glial fibrillary acidic protein identified spot on the insoluble fraction gels appeared to have lower molecular weights than those on the soluble fraction, and migrated further. This may be due to different isoforms or splice variants of the protein being expressed, or to breakage or modification of the protein in this fraction. Myelin basic protein was decreased in TAR (Figure 11). Myelin basic protein is a cationic protein found in myelin sheaths of both the central and peripheral nervous system of a wide variety of animals; among these are frogs, chickens, and a number of mammals including humans (Kornguth and Anderson, 1965). It is a cytosolic protein, and is involved in lipid-bilayer adhesion in compact myelin, as well as being associated with cytoskeletal processes (Review: Harauz et al., 2009).

The insoluble amygdala fractions were run once per sample (n=2). There were approximately 35 protein spots on these gels, but no significant differences between the two genotypes. The insoluble fraction of the ventral striatum did not yield a high enough protein concentration to be analyzed using 2DE in this study.



4A 4B

Figure 4: 2D gels for the soluble fraction of the amygdala. A representative TAR sample is on the left (4A) and TAP is on the right (4B). The protein identified from these gels was pyruvate kinase M2 (M2PK).

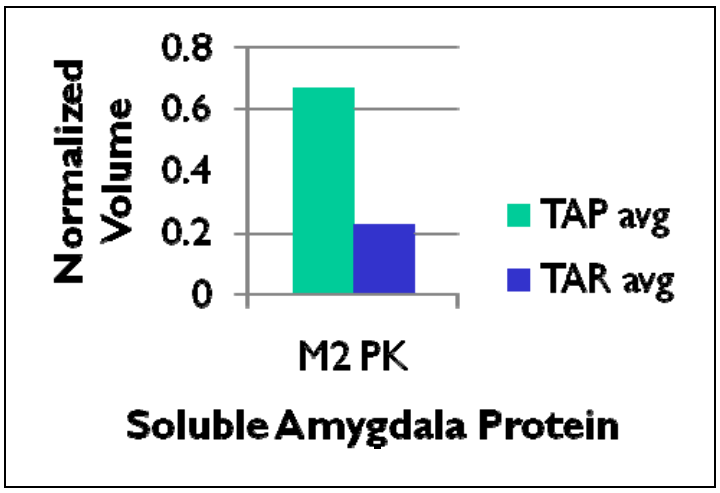
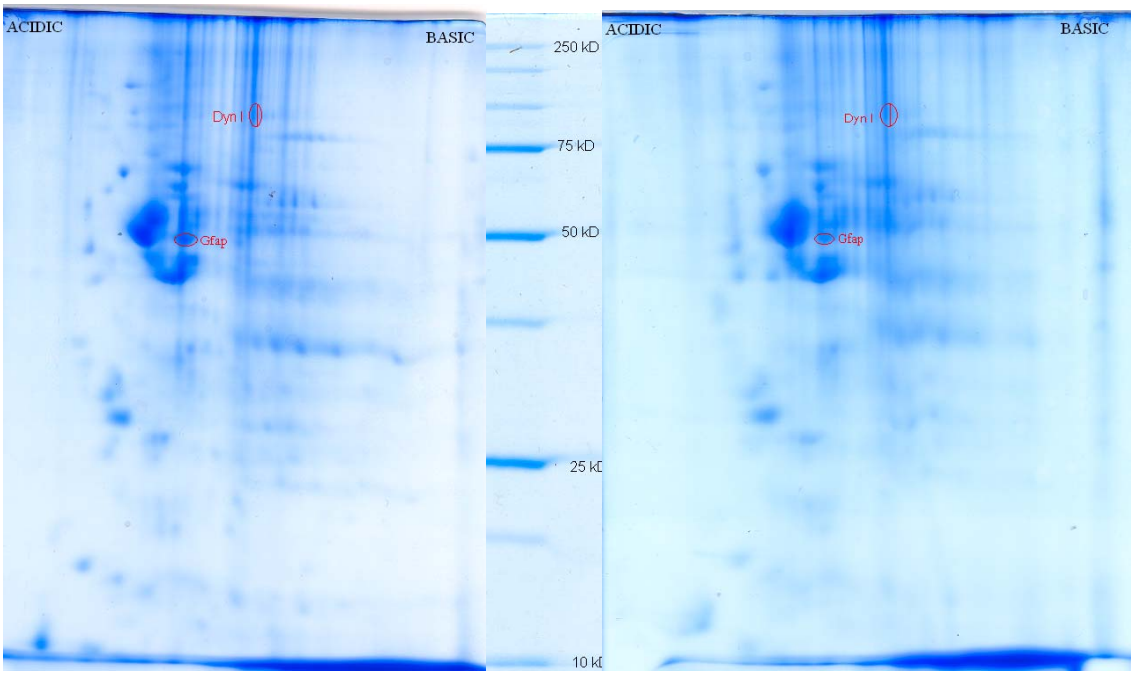


Figure 5: Normalized spot volumes of TAP and TAR for the soluble amygdala fraction gels. The protein identified from these gels was pyruvate kinase M2 (M2PK)



6A 6B

Figure 6: 2D gels for the soluble hippocampus fraction. A representative TAR gel is on the left (6A) and TAP gel is on the right (6B). The proteins identified from these gels were dynamin I (Dyn I) and glial fibrillary acidic protein – δ (Gfap).

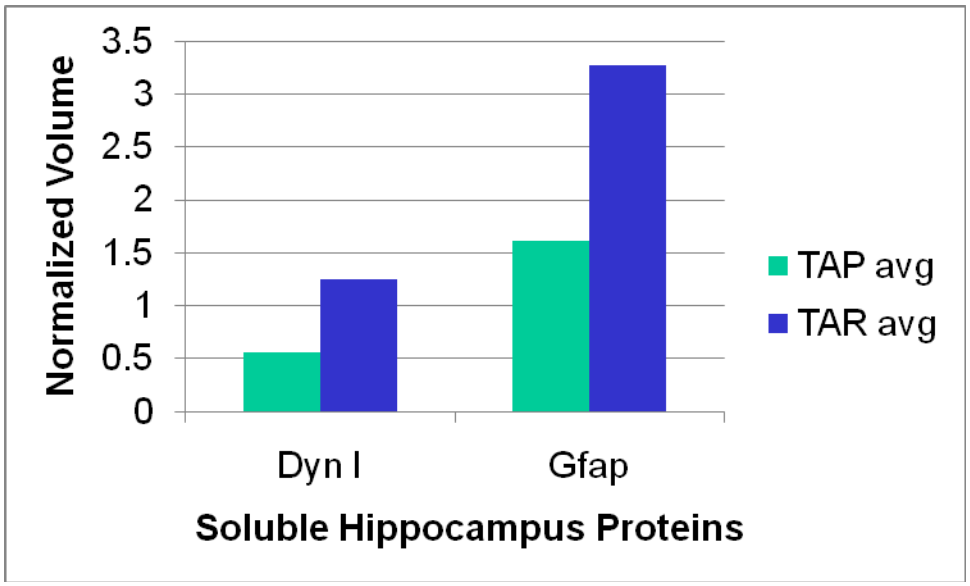
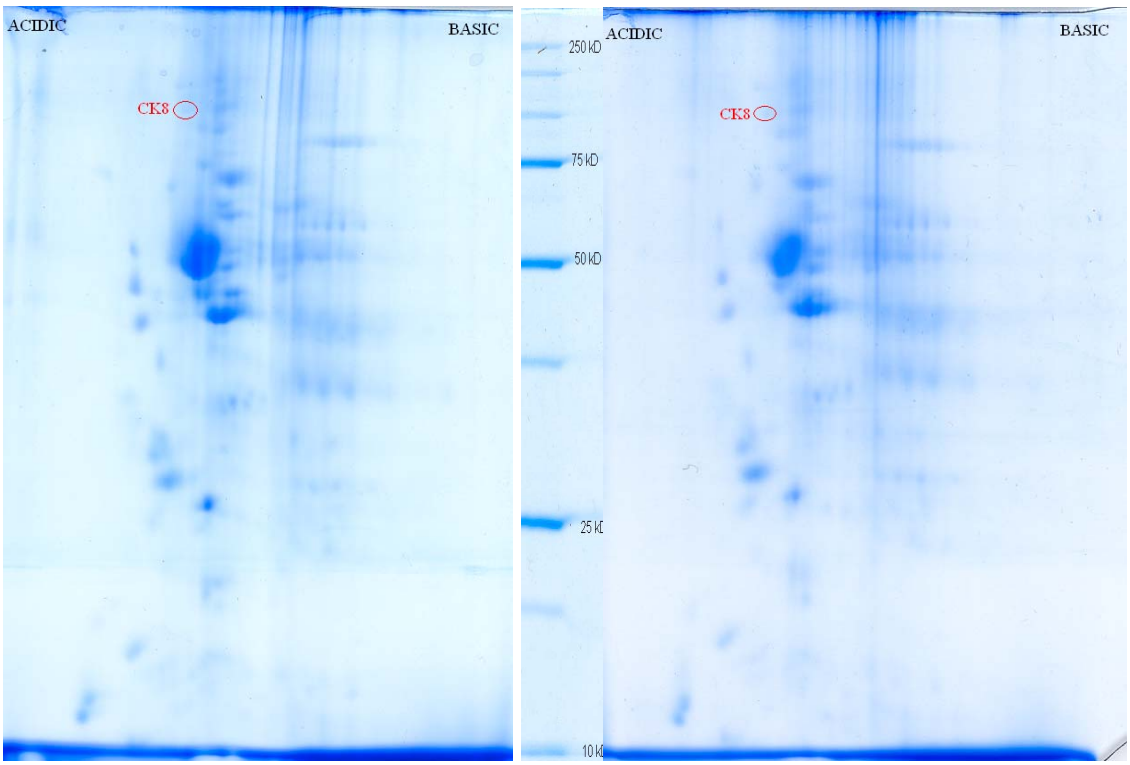


Figure 7: Normalized spot volumes of TAP and TAR for the soluble hippocampus fraction gels. Values for two dynamin 1 spots were added together. The two proteins identified from these gels were Dynamin I (Dyn I) and glial fibrillary acidic protein – δ (Gfap).



8A 8B

Figure 8: 2D gels from the soluble fraction of the ventral striatum. A representative TAR gel is on the left (8A) and a TAP gel is on the right (8B). The protein identified from these gels was cytokeratin 8 (CK8)

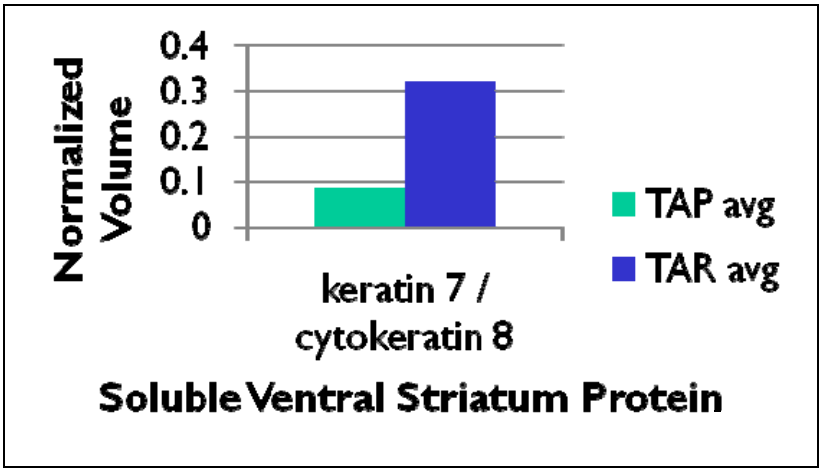
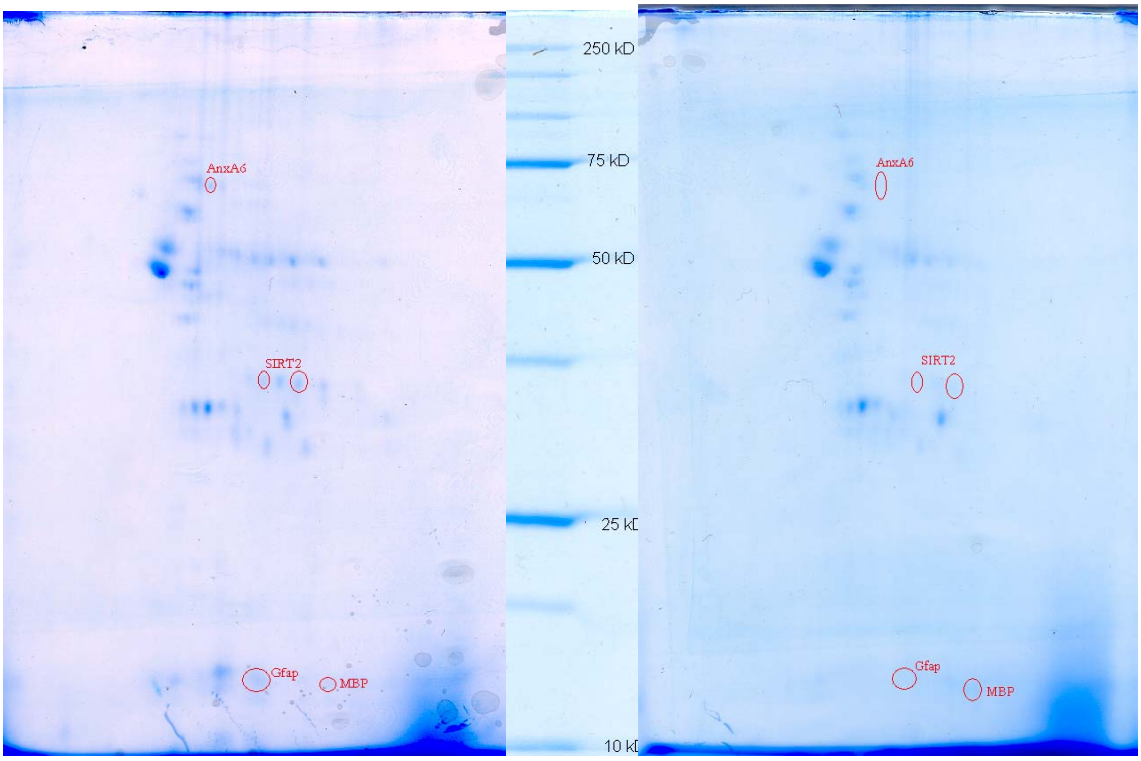


Figure 9: Normalized spot volumes of TAP and TAR for the soluble ventral striatum fraction gels. The protein identified from these gels was cytokeratin 8.



10A 10B

Figure 10: 2D gels from the insoluble hippocampus fraction. A representative TAR gel is on the left (10A) and a TAP gel is on the right (10B). The proteins identified from these gels were annexin A6 (AnxA6), sirtuin 2 (SIRT2), glial fibrillary acidic protein – δ (Gfap), and myelin basic protein (MBP).

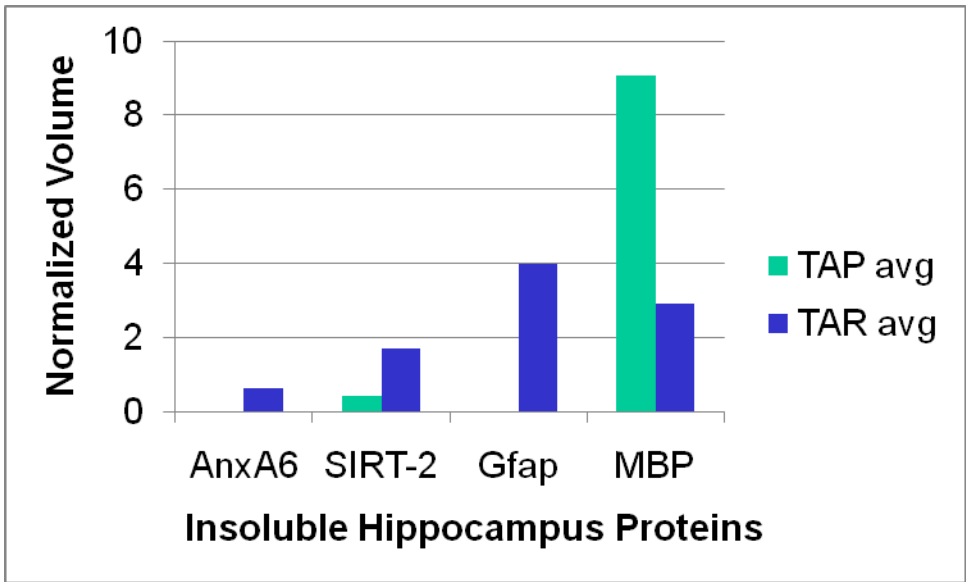


Figure 11: Normalized spot volumes of TAP and TAR for the insoluble hippocampus fraction gels. Values from the two sirtuin 2 spots were added together. The proteins identified from these gels were annexin A6 (AnxA6), sirtuin 2 (SIRT-2), glial fibrillary acidic protein – δ (Gfap), and myelin basic protein (MBP).

Table 1: Protein concentrations for TAP and TAR brain regions. Protein concentrations were determined by BIO-RAD RC/DC Protein Assay, utilizing the Lowry high sensitivity assay. Absorbance was measured at 750nm using Beckman Coulter DU800 spectrophotometer.

Brain Region	**Sample**	**Protein Concentration**
Soluble Hippocampus	TAPa	6.8704 mg/ml
	TAPb	12.8695 mg/ml
	TARa	13.8146 mg/ml
	TARb	16.5662 mg/ml
Soluble Amygdala	TAPa	5.7317 mg/ml
	TAPb	6.7955 mg/ml
	TARa	5.7351 mg/ml
	TARb	6.8569 mg/ml
Soluble Ventral Striatum	TAPa	1.8488 mg/ml
	TAPb	1.1525 mg/ml
	TARa	1.1975 mg/ml
	TARb	2.3075 mg/ml
Insoluble Hippocampus	TAPa	2.0639 mg.ml
	TAPb	4.3037 mg/ml
	TARa	4.4887 mg/ml
	TARb	6.4157 mg/ml
Insoluble Amygdala	TAPa	0.9925 mg/ml
	TAPb	1.1875 mg/ml
	TARa	0.8213 mg/ml
	TARb	1.9 mg.ml
Insoluble Ventral Striatum	TAPa	0.4075 mg/ml
	TAPb	0.45 mg/ml
	TARa	0.23 mg/ml
	TARb	0.5613 mg/ml

Table 2: Protein identifications, metabolic roles, and protein score confidence interval (CI). Data from Proteomics Core Laboratory, Medical College of GA. *Unnamed protein gi 56929 was updated in Genbank to the identification of Zinc Finger protein 41(Mus musculus)

Brain Region	Protein Name	Metabolic role	Accession #	pI	Protein Score CI %
Sol. Hip.	dynamin I	Cytoskeletal/ signaling	gi|18093102	6.32	100
Sol. Hip.	dynamin I	Cytoskeletal/ signaling	gi|18093102	6.32	100
Sol. Hip.	glial fibrillary acidic protein δ	cytoskeletal	gi|5030428	5.72	100
	Gfap protein	cytoskeletal	gi|57032786	5.35	100
Sol. Amyg.	*unnamed protein (gi|56929)		gi|56929	6.63	100
	M2 pyruvate kinase	Energy	gi|206205	7.15	100
Sol. V.S.	keratin complex 2, basic, gene 7	cytoskeletal	gi|14861854	5.67	99.09
	cytokeratin endo A; cytokeratin 8	cytoskeletal	gi|34869192	9.8	98.27
Ins. Hip.	annexin A6	Membrane-binding/Ca	gi|13994159	5.39	100
Ins. Hip.	sirtuin 2	Tubulin deacetylase	gi|55778661	6.67	92.95
Ins. Hip.	sirtuin 2	Tubulin deacetylase	gi|55778661	6.67	99.60
Ins. Hip.	glial fibrillary acidic protein δ	cytoskeletal	gi|5030428	5.72	0
	Gfap protein	cytoskeletal	gi|57032786	5.35	0
Ins. Hip.	myelin basic protein (18.5 kDa)	structural	gi|4454315	11.15	99.99
	myelin basic protein (21.5 kDa)	structural	gi|4454317	11.25	99.97

CHAPTER 4

DISCUSSION

Overview

This experiment utilized two-dimensional gel electrophoresis to measure the differences in protein expression between TAP and TAR rats. The brain sections analyzed were the hippocampus, amygdala, and ventral striatum. In these three sections of the brain there were a total of 10 protein spots expressed differentially between TAP and TAR that were identified.

Glial Fibrillary Acdic Protein - δ

Five of the proteins that were increased in TAR are involved in cytoskeletal structure and function, including glial fibrillary acidic protein – δ, dynamin I, annexin A6, sirtuin 2, and cytokeratin 8. Glial fibrillary acidic protein – δ occurs at lower rates in-vivo than glial fibrillary acidic protein -α, which is the main alternative splice variant of the GFAP gene, and is a component of intermediate filaments (Condorelli et al., 1999). When glial fibrillary acidic protein - δ is present at high concentrations, it forms aggregates with itself, which are easily disassociated. However, low levels of glial fibrillary acidic protein - δ (5-10%) can incorporate into filament structures with the more common glial fibrillary acidic protein-α without developing functional or structural problems (Perng et al., 2008). As such, it is possible that increased expression of glial

fibrillary acidic protein - δ could lead to decreased integrity of the intermediate filament cytoskeleton, which could, in turn, affect the CNS.

Glial fibrillary acidic protein is also affected by oxidative stress and other types of cellular injuries. Glial fibrillary acidic protein expression is increased in astrocytes when the CNS experiences an insult or injury, during a process called astrogliosis, or gliosis. The rapid increase in glial fibrillary acidic protein synthesis is utilized in the construction of intermediate filaments, which may play a role in healing or mediating injury (Eng et al., 2000). An increase in glial fibrillary acidic protein levels in response to injury is seen after exposure to or self-administration of several drugs of abuse. Cocaine and amphetamine intoxication both increase the expression of glial fibrillary acidic protein in rat brain tissue (Haile et al., 2001; Thomas et al., 2004). Lewis rats, a strain that has been selectively bred to self-administer high levels of drugs of abuse, do not show increased Glial fibrillary acidic protein in response to cocaine (Haile et al., 2001). In Wistar rats given access to 10% ethanol over 4 weeks (short-term exposure), 12 weeks (intermediate exposure), or 36 weeks (chronic exposure) different glial fibrillary acidic protein responses were observed in the brain. In the short and intermediate length alcohol exposure rats, there was an increase in glial fibrillary acidic protein expression, as expected from the insult of ethanol exposure. However, along with increased cell death of neurons, chronically exposed rats exhibited a decrease of glial fibrillary acidic protein – immunoreactivity (Franke et al., 1997). The chronic exposure to the damaging effects of ethanol in the CNS may lead to an attenuation of astrocyte response, and a down-regulation of glial

fibrillary acidic protein. It is also possible that a genetic predisposition to abuse drugs (such as that seen in the Lewis rat strain- Haile et al., 2001) may alter the response of glial fibrillary acidic protein to CNS insult or injury. If glial fibrillary acidic protein expression plays a role in mediating the response of brain tissue to drug-induced injury, it may also affect learned aversive responses. If this is the case, the increase in glial fibrillary acidic protein protein observed in the hippocampus of TAR rats compared to TAP may indicate a different mechanism of response to TA conditioning. This could occur through increased levels of glial fibrillary acidic protein – δ acting to destabilize the intermediate filament cytoskeleton and affecting gliosis, or through increased levels of total glial fibrillary acidic protein attenuating the cellular response to neural insult and injury. Consequently the cellular damage incurred through typical aversive stimuli, such as ethanol or other drugs of abuse, would be reduced.

Dynamin I and Annexin A6

Dynamin I and annexin A6 are both involved in the interaction of the cytoskeleton with the membrane during endocytosis, specifically of vesicle trafficking and recycling at clathrin-coated pits of the cellular membrane (Robinson et al., 1994; Tuma and Collins, 1994; Binns et al., 1999;, Kamal et al., 1998; Gerke and Moss, 2002). In the brain, dynamin I is present at relatively high, stable levels in the amygdala, striatum, and cortex (Faire et al., 1992). It appears to play a role in neuron growth and development. There is a steady increase in dynamin I expression from embryonic development up through early post-natal development, followed by a dramatic increase in expression during the

early growth of rat pups (post-natal day 15-23) (Faire et al., 1992; Cook et al., 1996). This increase in expression during a period of rapid neural growth and development is consistent with the role of dynamin I in neurite development (Torre et al., 1994). However, upon reaching adulthood, dynamin I levels remain high in neurons, which are no longer growing, pointing to other roles for this protein (Faire et al., 1992; Cook et al., 1996). Dynamin 1 in-vivo and in-vitro forms tetramers; this action is increased after phosphorylation by protein kinase C. The tetramer form of dynamin I binds to Ca^{2+} (Liu et al., 1996). Dynamin I exists in the cell at an equilibrium state between the monomer, a single dynamin I molecule, and the tetramer form (Binns et al., 1999). It is through the interaction of dynamin molecules to form tetramers that the GTPase activity may be regulated, as maximal GTP binding requires at least two dynamin I molecules (Tuma and Collins, 1994). The GTPase activity of dynamin I is also affected by binding with microtubules, and it is through dynamin self-associations that microtubule association reaches high levels (Tuma and Collins, 1994; Binns et al., 1999).

This protein appears to have many and varied functions within the cell. One series of experiments conducted by Cao et. al. (1998) used GFP-tagged dynamin genes to visualize the distribution of dynamins in the cell. Two of the eight splice variants of dynamin I were studied; while one of the variants was localized to cell membrane areas rich in clathrin, the other was located throughout the cytoplasm as well as at the golgi apparatus. These two splice variants differed by only 14 amino acids in the same inserted region, indicating

that the multiple splice variants of this gene could have varied roles throughout the cell.

Dynamin I was first identified as a GTP-dependent protein involved in receptor-mediated endocytosis at nerve terminals. Its activity was determined to be affected by associations with microtubules, phospholipids, and Ca^{2+} binding, as well as through phosphorylation (Review: Robinson et al., 1994). Though protein kinase C is the main enzyme that works to phosphorylate dynamin I (Liu et al., 1994, 1996), other types of kinases may also affect the action of dynamin I through phsophorylation (Earnest et al., 1996). Phosphorylation by protein kinase C increases the affinity of dynamin 1 for Ca^{2+}, leading to increased Ca^{2+} binding (Liu et al., 1996). After phosphorylation by protein kinase C, the GTPase activity of dynamin I is greatly increased, however the presence of Ca^{2+} attenuates this effect, and GTPase activity is reduced by as much as 50%. Ca^{2+} has no effect on the basal GTPase activity of dynamin I (Liu et al., 1996). The affinity of dynamin I for phospholipids binding is increased by Ca^{2+}: in the presence of Ca^{2+}, dynamin I migrates from the cytosol to the membrane, while the absence of Ca^{2+} results in an increase of cytosolic dynamin I (Liu et al., 1994, 1996). Only dynamin I in the cytolosol is available for phosphorylation by protein kinase C, after association with phospholipids in the plasma membrane the protein kinase C - phosphorylation site is not open (Liu et al., 1994). Calcium serves to regulate the activity of dynamin I, both through reducing the GTPase activity in the phosphorylated protein, and by leading to translocation of the protein from the

cytosol to the membrane, preventing phosphorylation, and maintaining basal GTPase activity.

Dynamin I is necessary for clathrin-coated pit invagination during vesicle recycling and receptor-mediated endocytosis, which plays a crucial role in nerve cell functioning (Review: Robinson et al., 1994). Likewise, annexin A6 is necessary for budding in some types of clathrin-coated pits. Annexin A6 binds with the protein spectrin in the cytoskeleton of the cell, which must be remodeled to allow for invagination of the clathrin-coated pits and the release of vesicles from the membrane (Kamal et al., 1998). Since vesicles are often involved in storing and releasing Ca^{2+} into the cell (Monastyrskaya et al., 2009), the regulatory effect of Ca^{2+} on both of these proteins that are intimately involved with vesicle endocytosis could critically affect intracellular Ca^{2+} homeostasis. Additionally, as calcium influx into the nerve terminal in response to the voltage change during the action potential is responsible for the exocytosis of neurotransmitters into the synapse, increased levels of these proteins may affect neurotransmitter release and nerve signal conductance (Sherwood et al., 2005). An increase in the intracellular Ca^{2+} level promotes annexin A6 binding to phospholipids (Gerke and Moss, 2002; Skrahina et al., 2007; Monastyrskaya et al., 2009). Under normal circumstances, high extracellular Ca^{2+} results in annexin A6 associating with the membrane, and leads to a reduction of roughly 20% of Ca^{2+} entry into the cell. Cells from annexin A6 knock-out mice exhibited elevated Ca^{2+} entry into the cell upon exposure to high extracellular Ca^{2+} concentrations (Monastyrskaya et al., 2009). However, membrane binding is also enhanced by a

drop in the intracellular pH or the pH of the extracellular environment, as well as in response to hypoxia (Monastyrskaya et al., 2008). Once annexins are bound to the membrane, they may self-assemble. Membrane-bound annexin mobility is low; annexin A6 has one of the lowest mobilities. This may affect the interactions of annexins on the membrane, and explain why assemblies consisting of different annexins are exceedingly rare to non-existent in vivo (Skrahina et al., 2007).

One of the characteristics of annexin proteins is the "annexin repeat", a sequence of 70 amino acids which is repeated and plays a role in membrane binding. Most annexins contain four copies of the annexin repeat which enables them to bind phospholipid membranes, however annexin A6 is unique in having eight (Avila-Sakar et al., 2000; Gerke and Moss, 2002; Grewal et al., 2010). The presence of eight annexin repeats gives annexin A6 a two-lobed structure; the conformation of which determines how annexin A6 interacts with membranes (Avila-Sakar et al., 2000; Grewal et al., 2010). The link between the two lobes appears to be quite flexible, allowing the lobes to form into an anti-parallel conformation; enabling the protein to bind to two membranes that are facing each other (Avila-Sakar et al., 2000).

In the brain and spinal cord, annexin A6 is found mostly in neurons, but not in astrocytes or oligodendrocytes. While it is found in the cytoplasm of the neural cell bodies, it is often located closely to the plasma membrane of axons, dendrites, and neurites. In addition to the role of annexin A6 in suppressing Ca^{2+} influx into the cell, it also negatively regulates K^{+} in sensory and spinal cord neurons (Naciff et al., 1996).

Both dynamin I and annexin A6 expression can be influenced by oxidative processes. In a study of cellular reprogramming using human macrophage cells, it was shown that exposing cells to oxidative stress in the form of ROS resulted in an increase in intracellular Ca^{2+} level. In response to oxidative stress, annexin A6 is released from lipid rafts into the cytoplasm. This leads to an increase in cytosolic Ca^{2+} as the bound Ca^{2+} is freed from annexin A6. Removing cholesterol from lipid rafts leads to annexin A6 redistribution into other parts of the cell, and decreases the Ca^{2+} response to oxidation (Cuschieri et al., 2005). Human hippocampal sections were studied to determine the effect of pathologies of neurons and glial cells on annexin expression. Annexin A6 was found to be associated with membranes of granulovacuolar bodies, which may play a role in neuron degeneration during Alzheimer's disease progression (Eberhard et al., 1994).

Dynamin may be affected by oxidative stress, though the studies exploring this link are looking at different models and methods of oxidation. A mouse-model of Down's Syndrome that over-expresses Cu/Zn SOD exhibited decreased levels of dynamin I in the hippocampus (Shin et al., 2004). While this could implicate superoxide or hydrogen peroxide as affecting the expression levels of dynamin, this study provides only corollary data, as the animal model was analyzing Down's Syndrome rather than oxidative damage explicitly. Dynamin levels are also affected by ethanol consumption. Rats were given chronic ethanol exposure through daily consumption over 15 months, and the effects on gene expression in the hippocampus were observed. Dynamin I had a decreased

expression of 1.5-fold in the chronically ethanol exposed rats compared to controls (Saito et al., 2002). Another study of hippocampal neurons found that dynamin I is necessary for the endocytosis-related activities of cannabinoid receptors (McDonald et al., 2007). This protein may also play a role in learning behaviors; in the striatum there is a great increase (3.2-fold) of dynamin I after initiation of certain long-term learning responses (Napolitano et al., 1999).

The increased levels of these two proteins observed in the hippocampus of TAR rats may indicate a role in taste aversion formation. Since both proteins are involved in receptor-mediated endocytosis and vesicle functioning, it is possible that some component of vesicle trafficking or intracellular communication may affect taste aversion conditioning. The effects of these two proteins on Ca^{2+} regulation and levels within the cell may also be of some importance. The increased expression of dynamin I in response to short term ethanol consumption (Saito et al., 2002) may indicate an advantage of over-expression seen in TAR rats, as they would already possess higher levels of this protein in the hippocampus. The release of annexin A6 into the cytosol, and its subsequent release of Ca^{2+} into the cell, in response to ROS may also confer a benefit; higher constitutive levels of annexin A6 could result in a larger influx of Ca^{2+} into the cytoplasm during oxidative stress, which could in turn affect neuron functioning.

Sirtuin 2

Sirtuin 2 interacts with the cytoskeleton through deacetylation of α-tubulin (North et al., 2003). In mice, sirtuin 2 expression levels increase during early

development, but then decrease to adult levels by about 1 month of age (Southwood et al., 2007). Post-translationally, sirtuin 2 activity is inhibited by cyclin-dependent kinase phosphorylation of a Ser-331 residue (Pandithage et al., 2008).

Sirtuin 2 is found in the cytoplasm of glial cells, specifically in oligodendrocytes and in myelin sheaths, where it is localized to microtubules (Southwood et al., 2007). Deacetylation of α-tubulin by sirtuin 2 represses growth and development of oligodendroglial cells, and may also inhibit cell adhesion and cell mobility (Li et al., 2007; Pandithage et al., 2008). Levels of sirtuin 2 are decreased in glioma cells, the most common type of malignant brain tumor (Hiratsuka et. al., 2003). Sirtuin 2 plays a role in mitosis control, which in turn may help to suppress growth of glioma tumor cells (North et al., 2003). Sirtuin 2 levels are decreased in mesial temporal lobe epilepsy, which is characterized by over-expression of α-tubulin (Yang et al., 2006).

An increase in levels of sirtuin 2, such as that seen in the TAR genotype, could have an effect on cell growth, mitosis, and proliferation, as well as cell-cell adhesion and mobility (North et al., 2003, Li et al., 2007; Pandithage et al., 2008). Though sirtuin 2 is normally found in the cytoplasm (Southwood et al., 2007), it was identified as part of the insoluble fraction in this study. It is possible that through its interaction with microtubules, or because of its location in the myelin sheaths, that this protein was not isolated from the cytoplasmic, or soluble fraction.

Cytokeratin 8

Cytokeratin proteins are involved in the cytoskeleton through their role as structural components of intermediate filaments. Certain cytokeratin proteins will form intermediate filaments with a number of others of the complementary type; cytokeratin 8, however, preferentially forms intermediate filaments only with its partner, cytokeratin 18. While most of the numerous cytokeratin genes have very similar structures, cytokeratin 8 has one fewer intron, it lacks the fifth of eight introns that are present in all the other cytokeratin genes (Krauss and Franke, 1990).

Cytokeratin 8 plays a crucial role in early development. Baribault et al. (1993) created cytokeratin 8 knock-out mice to elucidate the role of this protein, and those embryos that were homozygous for the cytokeratin 8 knock-out suffered mid-gestational lethality between days 12 and 13 of gestation. However, a small number of the embryos (about 6%) survived and lived to adulthood, though these individuals suffered from growth retardation resulting in reduced weights of 65-70%.

Cytokeratin 8 was down-regulated after incubation of colon carcinoma cells for 48 hrs in medium containing 20uM quercetin. Quercetin is a widely distributed flavonoid found in foods that may protect against several types of cancers, including colon. Quercetin treatment appears to attenuate the increased cytokeratin 8 expression often seen in cancer cells (Mouat et. al., 2005). Human breast cancer cells were used to study drug-resistance. Multi-drug resistant cell lines had greater cell adhesion, higher expression levels of cytokeratin 8, with

highest levels in the detergent-insoluble fraction. Blocking cytokeratin 8 decreased the cell adhesion of the multi-drug resistant cells, and also increased the sensitivity of the cells to several different drugs. From these results, it can be concluded that cytokeratin 8 plays a role in multi-drug resistance (Liu et al., 2008).

Cytokeratin 8 also responds to oxidation and ethanol insult. In human HepG2 cells, ethanol exposure (in the presence of polyunsaturated fatty acid and iron) led to a higher level of cytokeratin 8 and cytokeratin 18 (Bardag-Gorce et al., 2006). In rat alveolar epithelial cells, intermediate filaments were disassembled in response to hypoxia, cytokeratin 8 and 18 were decreased in hypoxic rats; hypoxia resulted in an increase in ROS production in the cells. Exposure to low oxygen or H_20_2 (hydrogen peroxide) resulted in decreased expression of cytokeratin 8 and 18. Over-expression of SOD2 protects cells from cytokeratin 8/18 decreases seen with hypoxia; increased SOD1 did not have an effect (Na et al., 2010). Proteomics was used to determine what protein expressions were changed in response to infection with *H. pylori*. Human gastric mucosal cells from patients with erosive gastritis, peptic ulcer or gastric cancer were analyzed. Decrease in cytokeratin 8 in patients infected with *H pylori*; protein was also oxidized. H. pylori infection causes oxidative damage through generation of ROS (Baek et al., 2004).

Since cytokeratin 8 levels are decreased in response to ROS, the increased levels seen in TAR rats could impart an advantage when presented with oxidative stress, such as that seen during ethanol consumption. Higher

constitutive levels could result in lower levels of cellular damage during oxidative stress. Such an effect could in turn attenuate taste aversion learning through reducing the aversive effects from compounds that generate oxidative damage in the CNS. Cytokeratin was the only protein spot identified from the ventral striatum. However, it is possible that a larger sample size would have led to identification of more spots.

Pyruvate Kinase M2

The two proteins that were lower in TAR than in TAP were pyruvate kinase M2 and myelin basic protein. Pyruvate kinase M2 is an enzyme in the glycolytic pathway, and was the only protein identified from the amygdala. Myelin basic protein is involved in membrane adhesion in compact myelin, and was identified from the insoluble fraction of the hippocampus.

During development, the more tissue-specific M1 isoform/isoenzyme often replaces M2, particularly in the muscle and brain tissues. (Mazurek et al., 2005, 2010) However, pyruvate kinase M2 is also present constitutively throughout the body, including in the fetal and adult brain (Gali and Bourdin, 1978).

Activated Pyruvate kinase M2 forms a tetramer, while inactivated Pyruvate kinase M2 is present as a dimer (Dabrowska et al., 1998; Mazurek et al., 2005). Pyruvate kinase M2 is subject to a high level of regulation by a number of different substances, many of which have little or no effect on other isoforms of pyruvate kinase. Pyruvate kinase M2 is allosterically regulated by fructose 1,6-bisphosphate in addition to rate effects from the substrates phospho-enol pyruvate and ADP (Dabrowska et al., 1998; Jurica et al., 1998). In the presence

of fructose 1,6-bisphosphate, pyruvate kinase M2 associates into the active tetramer formation (Dabrowska et al., 1998). The fructose 1,6-bisphosphate binding site of pyruvate kinase M2 is located in the 56-amino acid stretch that is the only difference between pyruvate kinase M1 and M2, which is why the M1 isoenzyme is not regulated by fructose 1,6-bisphosphate (Dabrowska et al., 1998; Christofk et al., 2008b). This same stretch of the enzyme also houses a phospho-tyrosine protein binding site, which affects the release of fructose 1,6-bisphosphate, and inhibits the action of pyruvate kinase M2 (Christofk et al., 2008b).The amino acid phenylalanine inhibits the activity of pyruvate kinase M2 by decreasing its affinity for phospho-enol pyruvate, however binding with liposomes containing phosphatidyl serine increases the activity of pyruvate kinase M2 (Schering et al, 1982; Dabrowska et al., 1998). Fructose 1,6-bisphosphate binding inhibits lipid binding of pyruvate Kinase M2, indicating that the phosphatidyl serine binding site may be near that of fructose 1,6-bisphosphate, or that fructose 1,6-bisphosphate may allosterically alter the lipid binding site (Dabrowska et al., 1998; Christofk et al., 2008b).

While pyruvate kinase M2 is replaced by M1 during development, during tumor formation the opposite is true, and the M2 isoform replaces M1 in cancerous cells (Mazurek et al., 2005, Christofk et al., 2008a). Pyruvate kinase M2 is present at the highest rate in the inactivated dimer form in cancerous tissues; the dimerized form is referred to as tumor-Pyruvate kinase M2 (Mazurek et al., 2005, 2010). Due to this change in expression and its release from tumor cells into the blood or GI tract, pyruvate kinase M2 may be used as a biomarker

for some cancers (Mazurek et al., 2010). The exact role of pyruvate kinase M2 in tumor formation is not completely understood, though it is known to increase the survival and proliferation of cancer cells. It may be involved in freeing up metabolic derivatives of glucose to be used for tissue synthesis, rather than energy production (Mazurek et al., 2005, 2010, Christofk et al., 2008a). Pyruvate kinase M2 is necessary for aerobic glycolysis, which is an important facet of tumor metabolism (Mazurek et al., 2005,2010). Cancer cells expressing M2 rather than M1 can grow at faster rates, and require less oxygen (Christofk et al., 2008a).

Investigators have explored a possible link of pyruvate kinase M2 and oxidative damage in the brain; in particular these studies have looked at age-related mental impairment and Alzheimer's disease. Both of these are marked by increased oxidative damage in various portions of the brain including the hippocampus and striatum (Butterfield et al., 2006; Poon et al., 2006). Poon et al. (2006) used proteomics to determine the effect that calorie restriction had on protein expression and oxidation. Calorie restriction appears to prevent some age-related impairment. In this study, a calorie restriction of 60% in aged rats led to a decrease in oxidative damage to proteins in all brain regions. It also resulted in a decrease in oxidation of pyruvate kinase M2 in the striatum. Mild cognitive impairment, which may be one of the first steps towards developing Alzheimer's disease, results in increased oxidative damage in the hippocampus. In individuals with mild cognitive impairment, hippocampal pyruvate kinase M2 was

oxidized at higher levels than in age-matched controls, and this oxidation resulted in a decrease in activity of 26% (Butterfield et al., 2006).

Pyruvate kinase M2 appears to be susceptible to oxidative damage, which may indicate why lower levels are seen in TAR compared to TAP rats. Lower constitutive levels of pyruvate kinase M2 could reduce the effects of oxidative stress through lessening the metabolic impact of lowered activity in this enzyme.

The lower levels of pyruvate kinase M2, coupled with increased levels of sirtuin 2 may indicate slower growth in certain cells in the TAR genotype. While sirtuin 2 is localized to glial cells (Southwood et al., 2007), pyruvate kinase M2 is a widely distributed isoform, and could be found in tissues throughout the body. Though only one of the three tissues analyzed in this study found differences of expression in this protein, further studies of pyruvate kinase M2 expression could determine whether there is a broader role for this protein in the maintenance or development of taste aversion.

Myelin Basic Protein

The other protein that was lower in TAR than TAP was myelin basic protein. Myelin basic protein is coded on the *Golli* gene, which was so named because it is the gene of oligodendrocyte lineage. The *Golli* gene contains at least three different gene products, one of which is myelin basic protein. There are additionally, several alternate splice variants of myelin basic protein, as well as a number of post-translational modifications to the individual myelin basic protein variants (Campagnoni et al., 1993; Harauz et al., 2009). The 18.5 kDa

isoform of myelin basic protein is the most commonly found in adult neural tissue (Review: Harauz et al., 2009).

Myelin is found in the central nervous system on the axons of oligodendrocytes, and in the peripheral nervous system as a component of Schwann cells. There are two types of myelin, compact and non-compact. Compact myelin is tightly compacted, and has very little cytoplasm; as such, cytosolic proteins in compact myelin interact with the plasma membrane to a high degree. Non-compact myelin however does contain cytoplasm, and thus has a higher level of separation between proteins that interact with the membrane, and those that are strictly cytosolic (Review: Kursula, 2008). Myelin basic protein accounts for 20-30% of myelin protein by weight (Min et al., 2009). It is a cytosolic protein, and is involved in lipid-bilayer adhesion in compact myelin. The most common form of myelin basic protein in the cell has not undergone post-translational modification, and possesses a charge of around +19 -20. These charges (basic residues) are spread throughout myelin basic protein, which leads to its role in membrane adhesion, stretching across membranes to bind them together (Kursula, 2008; Harauz et al., 2009; Min et al., 2009).

Myelin basic protein, in addition to its role in cytosolic membrane adhesion of myelin, is also associated with cytoskeletal processes, and binds with actin, tubulin, microtubules, microfilaments, calcium and calmodulin. Myelin basic protein is an intrinsically disordered protein, however it will form secondary structures in response to binding with these different proteins, lipids, or other elements (Libich and Harauz, 2008; Harauz et al., 2009). When bound to an

artificial myelin membrane, Zn^{2+} and Cu^{2+} will bind to, and alter, the secondary structure of myelin basic protein (Baran et al., 2010; Smith et al., 2010).

The level of myelin basic protein in myelin is crucial to its proper functioning: too little and there is a loss of function; too much and water is pushed out of the cytoplasmic gap between membranes, leading to the formation of a gel and loss of adhesion (Min et al., 2009). Studies of myelin basic protein interactions with lipid bilayers using lipid films have revealed that changes in surface pressure can also affect the function of this protein. Under normal or low surface pressures the cationic myelin basic protein associates with anionic lipids, penetrating into the membrane. However at high surface pressure myelin basic protein maintains a distance from the lipid layer (Rispoli et al., 2007). Myelin basic protein may be involved in cell signaling, as well as cytoskeletal anchoring; it is possible that these processes take place during myelination of axons, prior to the compaction of myelin, when there is still cytoplasm present. It may then switch roles to focus more on its role of membrane adhesion after the compaction of the myelin and the removal of most of the cytoplasm (Harauz et al., 2009).

Demyelination is involved in several disease processes, such as multiple sclerosis in humans. Myelin basic protein has been used to induce allergic encephalomyelitis in animal models of multiple sclerosis (Hashim 1978). As part of demyelination, or as a response to stress, myelin basic protein is released from cells (Hashim, 1978; Wang et al., 2004). One area of study focusing on this effect is the etiology of stress-disorders following severe injury, such as that seen

in post-traumatic stress disorder. Wang et. al. (2004) studied the effect of high-speed and low-speed peripheral trauma (gunshots to the thighs) on myelin basic protein levels in the cerebro-spinal fluid, hippocampus, and hypothalamus of dogs. In this study, high-speed trauma resulted in increased myelin basic protein in the cerebro-spinal fluid, hypothalamus, and hippocampus, while low-speed trauma resulted in increased myelin basic protein in the cerebro-spinal fluid and hypothalamus only. Myelin basic protein mRNA was increased in all three locations. Neuron degradation and demyelination were observed in the hypothalamus and hippocampus.

Myelin basic protein may also play a role in learning. Atkins et al. (1997) studied the link between myelin basic protein phosphorylation and long-term potentiation in the hippocampus. Long-term potentiation is an increase in synaptic efficiency which may be indicative of certain types of learning and memory. Myelin basic protein phosphorylation is increased in response to long-term potentiation. High-frequency stimulation of CA1 neurons in the hippocampus resulted in myelin basic protein phosphorylation by protein kinase C. However, the addition of SOD, catalase, and nitric-oxide syntase inhibitor blocked this phosphorylation, both together, and when added individually (Atkins et al.,1999). These results would appear to indicate that ROS can affect myelin basic protein, through attenuating its phosphorylation by protein kinase C; Atkins et al. (1999) suggested that this affect may be through neuron-glia communication, with ROS acting as intercellular messengers.

The role of myelin basic protein in myelination, as well as its usefulness as a biomarker of neural degeneration and stress, emphasizes the importance of this protein in neural function and its responsiveness to neural insult and/or injury. Highlighting the importance of this protein is the fact that too little or too much of it in myelin can result in loss of function (Min et al., 2009).

CHAPTER 5

CONCLUSIONS

The TAP and TAR strains are no longer maintained. Though this deprives us of further research opportunities with this particular model, there are many other prospects for utilizing the results of this study. There are several animal models available for studying the effects of drugs of abuse and ethanol preference. As seen in Liu et al. (2009), experimental lesions can also be used to study the effects of specific brain regions on TA formation. Predispositions to TA conditionability are widespread, and can be seen in rat pups (Arias et al., 2009). This would be easy to exploit for further research, with or without instituting a new breeding program.

The data presented here are only preliminary due to the limited number of samples available for this study. Although the observed densitometric differences in protein expression were not statistically significant, the identified proteins may ultimately be directly involved in the development of taste aversion resistance and thus are molecular targets for future research concerning the development of this phenomenon.

Several of the proteins found to be differently expressed between TAP and TAR are involved in calcium homeostasis, or are affected by intracellular calcium levels. Dynamin I and annexin A6 are both involved in vesicle trafficking

at the membrane, and both respond to intracellular calcium levels by binding with the plasma membrane (Liu et al., 1994, 1996; Gerke and Moss, 2002; Skrahina et al., 2007; Monastyrskaya et al., 2009). Both dynamin I and annexin A6 were increased in TAR rat hippocampus, which may indicate a difference in calcium levels or response, particularly since annexin A6 membrane binding leads to decreased influx of Ca^{2+} into the cell (Monastyrskaya et al., 2009). Of particular interest when looking at learning, such as conditioned taste aversion is the role of Ca^{2+} in nerve signal transduction, through the release of neurotransmitter into the synapse. It is possible that increased levels of these two proteins could affect the response to nerve signals at the pre-synaptic axon terminal. Since the hippocampus is involved in the transfer of short-term to long-term memories, as well as in forming connections between related stimuli (Sherwood et al., 2005), such an effect could play a role in the decreased TA conditionability seen in TAR rats. Further research that specifically measured the levels of these, or other proteins involved in nerve signal transduction, in the hippocampus could demonstrate the role that these proteins play. Since the original TAP/TAR strains are no longer available for research, other models of TA would have to be used, perhaps utilizing strains such as the P/NP alcohol preference rat strain.

One of the two proteins present at lower levels in TAR than TAP was pyruvate kinase M2, a glycolytic enzyme which catalyzes the production of ATP. As the rate – limiting enzyme in glycolysis, this enzyme is intimately connected with energy production. High concentrations of fructose1,6-bisphosphate activate the enzymatic activity of pyruvate kinase M2, resulting in energy production

through glycolysis. Under normal circumstances the enzyme is inactive, leading to a build-up of glycolytic intermediate products which can be used for macromolecular synthesis within the cell (Dabrowska et al., 1998; Mazurek et al., 2005, 2010; Christofk et al., 2008b). Since the only proteins identified in this study were those exhibiting differences in expression, the M1 isoform of pyruvate kinase was not detected. The current interpretation of the data, therefore, is that the M2 isoform is the only one affected by taste aversion resistance. In the future, increasing the protein loading concentration for 2DE or performing mass spectrometry on all of the proteins spots within the gels may allow for determining the ratio of the M2 to M1 isoforms, and the full effect of taste aversion resistance on pyruvate kinase.

The possible role that energy availability in the brain may play in taste aversion, especially in light of the possible differences in Ca^{2+} that the proteins dynamin I and annexin A6 indicate, warrant further study. A pyruvate kinase M2 – knockout model could be used to test the effects of this protein on taste aversion conditionability. This difference in expression levels of pyruvate kinase M2 was identified in the amygdala, which is a brain region responsible for forming a connection between conditioned and unconditioned stimulus (Sherwood et al., 2005). It is possible that the difference in energy metabolism represented by the lower expression of pyruvate kinase M2 in TAR may affect the association of conditioned and unconditioned stimuli, and may play some role in the reduced acquisition of TA seen in TAR. However, not all conditioned – unconditioned stimulus pairings are affected, as shock-motivated environmental

avoidance and maze learning were not diminished in TAR compared to TAP (Hobbs, 1993; Elkins, 1986). It is possible that the affected pathways are specific to TA formation, rather than affecting all conditioned – unconditioned stimuli learning. Using a technique such as immunoreactive staining to determine which cell types and regions within the amygdala may exhibit the altered levels of pyruvate kinase M2 in conjunction with TA conditionability might shed light on the exact pathways affected by this behavioral difference.

In the absence of the TAP/TAR strain, knock-out models could be developed to determine the effects of each of the proteins identified in this study. Likewise, radioimmuno-assays could be used to determine the levels of each of these proteins in the brains of other strains that exhibit differential TA responses.

In addition to the lack of statistical significance brought about by the low sample size available for this study, there was also no opportunity to determine the effect of acute vs. chronic ethanol consumption on protein expression using the TAP/TAR rats. All animals used in this study were ethanol naïve; there is now no opportunity to compare their brains before and after exposure to different levels of ethanol. It is possible that such a comparison would have yielded even more information about the possible neural mechanisms and pathways affected by TA conditioning. However, the proteomic techniques utilized in this study identified several differentially expressed proteins between the two strains, providing possible avenues of further research into taste aversion.

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