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Role of Glia in the Development of Ethanol Tolerance

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science
in
Quantitative and Systems Biology

by
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DEDICATION

Firstly, I dedicate this thesis to my best friend, Victoria Hilt. Torie you are my rock. You have been a source of encouragement and support when I have needed it the most. You have helped me through some of my darkest days, and are always able to remind me of my strength when I forget.

Meet me in Montauk

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ABSTRACT OF THESIS

Despite widespread abuse, high socioeconomic costs, and substantial research investment, the basic mechanisms of alcohol action on the brain remain poorly understood. This is partly due to the physiological complexity of alcohol's effects and the long term progressive nature of alcohol use disorders (AUDs). Further, mammalian models of AUD endophenotypes require high levels of resources and time. One approach that has promise is to use invertebrate model organisms to understand the molecular and cellular mechanisms of behavioral adaptations to acute ethanol exposure. The fruit fly Drosophila, is a classic model organism for defining the molecules and neural circuits that drive animal behavior. The molecular makeup of the fly brain is remarkably conserved with that of mammals. Moreover, both flies and humans have a long history of association with alcohol, suggesting that behaviors like craving, drinking, and reward are coded similarly. Indeed, dopamine signaling underlies the hyperactivating and rewarding properties of ethanol across species. Flies, like humans, become inebriated, develop ethanol tolerance, ethanol preference, and ethanol reward associations, and they show signs of withdrawal. Many of these are adaptations to ethanol exposure that are forms of behavioral plasticity. How ethanol behavioral plasticity differs from non-addictive forms is key to understanding why some substances are abused. The goal of the research for this thesis was to ask if glial cells, like neuronal cells, promote behavioral plasticity induced by acute ethanol. Glial cells perform surprisingly diverse functions in the brain, including information transmission whose regulation is key to behavioral plasticity. A survey of the Drosophila glial types uncovered roles in ethanol tolerance for two types, the astrocytes that contact and regulate neuronal synapses, and the perineurial cells that form the outer surface of the blood-brain barrier. Dysregulation of glutamate homeostasis in astrocytes renders flies sensitive to acute inebriation and decreased ethanol tolerance. These ethanol phenotypes correlate with others that are early signatures of neurodegeneration caused by glutamate excitotoxicity. Perineurial cells show morphological change that correlated with reduced actin organization following acute ethanol exposure. This morphological change required Akap200, an adaptor protein that coordinates protein kinase A, protein kinase C, calcium, and actin at the perineurial plasma membrane. Loss of Akap200 either globally or specifically in the perineurium decreases ethanol tolerance development, as does disruption of many of the molecules that interact with Akap200. These Akap200 dependent functions appear to be occurring at the time of ethanol exposure. These findings indicate an active signaling role for the blood-brain barrier in the development of ethanol tolerance, and they imply that the barrier and neurons communicate to promote behavioral plasticity.

CHAPTER 1: INTRODUCTION

1.1 Dissertation Statement

Glial cells in the brain regulate ethanol tolerance development, demonstrating that both glia and neurons control behavioral plasticity induced by drugs of abuse.

1.2 Ethanol Pharmacology and Neuronal Plasticity

Ethanol is the most widely used and frequently abused addictive drug. The term "alcohol use disorders" (AUDs) describes the range of pathological effects of ethanol, from abuse, to dependence, to addiction. Amongst drugs of abuse, ethanol is unique: humans have a very long association with the drug, brewing it since ancient times to make drinking water safe and for socializing. Further, the majority of all humans have had an alcoholic drink, and many enjoy drinking over a lifetime without ever developing an AUD. However, the cost to society is high: for example, well over 50% of all emergency room visits involve alcohol in some form (Sacks et al., 2015). Furthermore, the current best treatment - group therapy like Alcoholics Anonymous - is only slightly more effective than no treatment at all. The relapse rate is astoundingly and unacceptably high. Our partial understanding of how ethanol affects brain function and behavior has seriously hampered the development of medications and behavioral treatments for AUDs.

The actions of ethanol can be classified as acute and the plasticity of those responses with repeat drinking, longer term chronic, withdrawal, and relapse. Whereas chronic ethanol intake is most directly tied to the development of AUDs, its acute actions provide the clearest window into how ethanol pharmacology impacts the cellular and molecular properties of the brain. A basic, and likely true, assumption of our work is that these direct actions of acute ethanol cause changes that prime the brain for the longer-term effects of the drug, therefore providing a needed simplifying window into the complex biology of addiction.

How acute ethanol intake causes adaptations in behavior is what we seek to understand. One of the simplest forms of neuroadaptative change is the development of tolerance. Ethanol tolerance can be defined as the acquired resistance to its aversive and pleasurable effects that facilitates increased ethanol intake (Fadda and Rossetti, 1998). For example, if three drinks in succession causes unconsciousness, three drinks a day later may only cause inebriation. Ethanol tolerance facilitates increased ethanol intake, a clarion risk factor for developing an AUD.

Ethanol tolerance has multiple forms with differing underlying biology. Tolerance is classified as acute, rapid, and chronic. Acute tolerance develops within a bout of drinking. Acute tolerance is due in part to changes in the conductance properties of neurons through changes in potassium channel quantity and activity (Lewohl et al., 1999). Rapid tolerance develops after alcohol from the first drink has been completely

metabolized, over the course of hours or days. This is a longer lasting form of tolerance, and is the subject of the studies described in this thesis. Chronic tolerance develops after repeated exposures to alcohol and is the result of multiple neurological and physiological adaptations. All these forms of tolerance can be either dispositional or functional: changes in ethanol metabolism are termed dispositional, and changes in neuronal properties are termed functional.

1.3. Drosophila as a Model for Ethanol Behavioral Plasticity

Animal models are particularly useful for defining the molecular and cellular mechanisms of complex biology and pathology (Barkley-Levenson and Crabbe, 2012). The most commonly used models for AUD endophenotypes are mice and rats. The fruit fly Drosophila is also a well-established model for identifying the molecular mechanisms of both simple and more complex behavioral responses to ethanol. Drosophila, like humans, have a long history of alcohol intake. Their favored source of food is rotting fruit that can contain up to 5% ethanol concentration (Dudley, 2000). Furthermore, ethanol is a naturally occurring molecule commonly found in nature that is not much larger than water, suggesting that its basic molecular interactions are conserved across organisms.

Drosophila have been used for over a century to discover the basic mechanisms of genetics, of cell structure and function, and the signaling mechanisms that let multicellular organisms develop and function (Chao et al., 2017). Drosophila genes are well conserved with those of mammals, and nearly all molecules that let the nervous system develop and work are found in Drosophila. Advances in transgenic technology lets researchers easily manipulate genes and cellular properties in highly cell-type and temporally specific manners. For example, RNA interference (RNAi) against of a specific gene allows tight spatiotemporal reduction of that gene to test its role in a cellular or behavioral process, like ethanol tolerance. Transgenic technology also made accessible exquisite details of the neuroanatomy, allowing for highly precise connectivity to be studied. All these technologies were used in the studies described in this thesis.

Drosophila exhibit behavioral responses to ethanol that broadly mirror those seen in mammals (Rodan and Rothenfluh, 2010). Drosophila given an acute ethanol exposure show progressive behavioral responses as the ethanol dose increases. Low doses stimulate locomotion, akin to hypersociality and behavioral disinhibition in humans. As the dose increases, Drosophila become progressively more incoordinated and then sedated. Sedated flies wake up and regain normal behaviors as ethanol is metabolized by a conserved metabolic pathway. Flies that recover will show sensitization to the locomotor activating effects and tolerance to the incoordinating and sedating effects of ethanol. Flies given continuous access to a choice of food and food plus ethanol will initially avoid the ethanol, but over the course of days they will develop a preference for it. This ethanol preference is for its pharmacological and not its caloric value (Devineni and Heberlein, 2009; Peru Y Colón de Portugal et al., 2014; Xu et al., 2012).

Perhaps even more compelling, a low dose of ethanol presented with a behaviorally neutral odor cue will later become attracted to that cue when presented alone (Kaun et al., 2011). This indicates that flies find ethanol rewarding. Finally, flies show signs of ethanol withdrawal after chronic ethanol exposure, that, like in higher organisms, is due in part to inhibition of GABAergic inhibitory interneurons (Ghezzi et al., 2014).

Evidence for molecular parallels for ethanol action in flies and mammals is growing. One of the best examples is dopamine, which plays a critical role in the reinforcing/rewarding properties of all known drugs of abuse. In mammals, ethanol exposure causes dopamine release from ventral tegmental area neurons into the nucleus accumbens. Rodents and other mammals with blocks to this dopamine response fail to show locomotor stimulation or associate external cues with the drug (Berridge and Robinson, 1998). In flies, genetic blockades of specific dopamine circuits has the same effect: reduced locomotor activation and loss of ethanol reward (Kaun et al., 2011; Kong et al., 2010a). Other molecular and circuitry parallels are also emerging, with new advances occurring in both mammals and Drosophila. Thus, not only are behavioral responses to ethanol conserved across phyla, but also the molecular mechanisms driving those behaviors.

The molecular mechanisms of ethanol tolerance are partly understood in Drosophila. The main behavioral paradigm used involves the induction and measurement of rapid tolerance, however flies also develop acute and chronic tolerance (Berger et al., 2004). Rapid tolerance in Drosophila is functional, meaning that tolerance is due to ethanol altering the function of the nervous system and not altering ethanol metabolism (Scholz et al., 2000). Importantly, while ethanol sensitivity and tolerance are correlated in wild-type, they are easily separable genetically, indicating that their underlying mechanisms are distinct (Devineni et al., 2011). From here on the term “tolerance” will refer to “rapid tolerance”. Typically, flies are given a just sedating dose of ethanol over the course of about ½ hour, are allowed to recover and completely metabolize accumulated ethanol for 3.5 hours, and then are given a second, identical ethanol exposure. There are a number of ways to measure ethanol tolerance, including loss of postural control, loss of the righting reflex, and recovery of the righting reflex during the recovery period (Rodan and Rothenfluh, 2010). Tolerance is induced by 2 hours after exposure, and lasts at least one day. Classic genetic mutant screening often coupled with gene expression studies have identified several genes involved in tolerance development, including the Hangover RNA binding protein, the Homer adaptor protein, the Sirtuin histone deacetylase, and the Slowpoke inwardly rectifying potassium channel, all of which are conserved by sequence in mammals. Indeed, to date a list of about 50 genes have been implicated in tolerance development with varying degrees of evidence quality (Ghezzi et al., 2013; Kong et al., 2010b; Morozova et al., 2006; Urizar et al., 2007).

Comparatively less is known about the cellular circuitry for ethanol tolerance. The current best evidence points to two well-studied regions of the Drosophila brain, the mushroom bodies and the central complex ellipsoid body. The mushroom bodies are

critical centers for associative learning and memory, but they also perform some non-associative or innate functions (Guven-Ozkan and Davis, 2014). Inactivation of mushroom body synaptic output decreases ethanol tolerance, as does reduction of Sir2 expression in specific mushroom body lobes (Engel et al., 2016). The Homer postsynaptic adaptor protein is needed in the ellipsoid body, a part of the central complex that is thought to coordinate sensory information with motor output (Seelig and Jayaraman, 2015; Urizar et al., 2007). How these regions communicate and their specific roles in the development of tolerance remains to be discovered. To the credit of Drosophila research, even less is known about the circuitry of ethanol tolerance in mammals.

1.4. Role of Glia in the Actions of Ethanol

Glia are the main non-neuronal cells of the nervous system, however current estimates place them at a 1:1 ration with neurons (Azevedo et al. 2009, von Bartheld et al 2016). There are three main types of glia in the mammalian brain: astrocytes, oligodendrocytes, and microglia. Oligodendrocytes ensheath neuronal axons to enhance action potential conductance and to provide electrical isolation. Microglia perform immune-like functions in the brain, engulfing non-self, carrying out inflammatory reactions, and removing cellular debris. Astrocytes are star-shaped cells that infiltrate all the neuronal synaptic regions of the brain. They also make extensive contact with the endothelial cells that form the physical and chemical barrier properties of the blood-brain barrier. Astrocytes are remarkably versatile, performing metabolic, homeostatic, and structural roles. The major metabolic roles of astrocytes are transport of glucose and lactate from the circulation to neurons, and uptake of potassium and neurotransmitters following synaptic release, thereby cleaning up the extracellular space to provide for clean signal propagation. One of the best characterized properties of astrocytes is the regulation glutamate homeostasis. Synapses release glutamate from synaptic vesicles, loaded by VGlut, a vesicular glutamate transporter, as a neurotransmitter. Astrocytes take up this glutamate to clear the synaptic cleft via excitatory amino acid transporters (EAATs). Glutamate is then converted into glutamine in astrocytes and released back to the presynaptic neuron, where it is taken up through the EAAT2 transporter. Once inside the neuron, glutamine is converted into glutamate and loaded into vesicles to repeat the cycle (Danbolt, 2001).

Astrocytes also comprise part of the blood-brain barrier in mammals, along with endothelial cells, and pericytes (Obermeier et al., 2013). Their direct apposition on the barrier endothelium is critical for two well studied functions, the aforementioned nutrient transport, and the regulation of cerebrovascular blood flow (Bélanger et al., 2011). This latter function is coupled to their role in glutamate homeostasis: glutamate conversion produces small molecules that can cause either vasodilation or vasoconstriction: increased glutamate uptake is a sign of neuronal activity, and results in vasodilation, bringing increased oxygen and nutrients to the active area.
Recent findings demonstrate that astrocytes are also important for the regulation of synaptic plasticity that underlies most forms of behavioral plasticity (De Pittà et al.,

2016). The neuronal process of long-term depression that decreases activity at a synapse requires an intercellular communication pathway that travels from the postsynaptic to the presynaptic neuron through the closely apposed astrocyte (Min and Nevian, 2012). Astrocytes also perform a regulatory role in memory stability through the neurotransmitter adenosine (Orr et al., 2015).

Drosophila glia are less studied than in mammals. Early findings indicate that Drosophila glia perform many of the same functions as their mammalian counterparts, however some functions are distributed differently. There are three main classes of Drosophila glia: surface-associated, cortex, and neuropil-associated. The Drosophila blood-brain barrier is composed of the surface-associated glia, and it forms a physical and chemical barrier between the brain and the circulating hemolymph. The surface glia are two closely apposed layers of glia: the outermost perineurial glia interfaces directly with the circulating hemolymph, while the subperineurial glia are located just beneath where they form both the physical and chemical barrier (Awasaki et al., 2008; Hindle and Bainton, 2014). Like the mammalian barrier, the Drosophila barrier is selectively permeable to a variety of proteins and small molecules, including sugars that provide the nervous system with energy (Volkenhoff et al., 2015). My research (Chapter 4) provides some of the clearest evidence to date that the barrier also generates signals to transmit information between the periphery and the central nervous system.

Little is known about the cortex glia that surround the neuronal cell bodies or the ensheathing glia that insulate neuropils from one another. Interestingly, the ensheathing glia appear to perform some microglia functions, in particular they are phagocytic and help drive neuronal remodeling during metamorphosis (Doherty et al., 2009).

Drosophila astrocytes are apposed to synapses and they share a similar morphology to their mammalian counterparts. Indeed, Drosophila astrocytes perform metabolic, homeostatic, and structural roles. Importantly, the glutamate homeostasis function in Drosophila and mammalian astrocytes is identical (Freeman et al., 2003). Furthermore, Drosophila astrocytes regulate sleep, circadian rhythms, and also modulate neuronal function (Farca Luna et al., 2017; Ma et al., 2016; Ng et al., 2011).

Ethanol affects glia in both physiological and pathological conditions. Short-term exposure to physiologically-relevant ethanol concentrations increases the levels of a glial-specific cytoskeletal protein (Blanco and Guerri, 2007; Bull et al., 2015; Goodlett et al., 1993). Studies with binge and chronically drinking rodents reveal astrogliosis and changes in glial density following moderate levels of ethanol intake (Bull et al., 2015; Evrard et al., 2003; Fattore et al., 2002; Goodlett et al., 1997; Miguel-Hidalgo, 2006). Long term changes in the expression levels of glial markers and in the proliferation of oligodendrocytes and microglia after cessation of ethanol self-administration suggests that glia may also contribute to the long-lasting effects of ethanol on behavior (Evrard et al., 2006; He et al., 2009; Nixon, 2008).

Drosophila glia have been implicated in the sensitivity to drugs of abuse, including ethanol and cocaine (Bainton et al., 2005; Hoxha et al., 2013; Ng et al., 2011). The strongest study to date demonstrated that mutations of the G protein-coupled receptor Moody lead to disruption of the physical barrier and decreased ethanol sensitivity and increased cocaine sensitivity (Bainton et al., 2005). No studies are published that link any type of glia in either Drosophila or mammals to the behavioral adaptations induced by ethanol. The one potential exception is for glutamate transmission and homeostasis.

Glutamate, the major excitatory neurotransmitter in the brain, is intimately tied with the actions of ethanol. Classic studies showed that alcohol interacts with glutamate receptors, blocking their function (Lovinger et al., 1989). This is part of the depressant effect of intoxication, decreasing brain activity at higher ethanol doses. Lower doses of ethanol potentiate glutamate signaling, causing greater neurotransmitter release and contributing to the hyper sociality that accompanies its euphoric effects. The enduring adaption of glutamate receptor signaling is implicated in the development of ethanol dependence, tolerance and addiction (Chandler, 2003). In Drosophila, the protein Homer, that interacts with glutamate receptors, regulates ethanol tolerance, suggesting that glutamate signaling is important for the behavioral effects of ethanol in flies (Urizar et al., 2007).

Disruption of glutamate homeostasis through drugs of abuse implicate astrocytes as potential regulators of drug action. Work in rat astrocyte in culture and in the nucleus accumbens have shown that cocaine exposure leads to decreased expression of xCT and GLT-1, astrocyte-specific glutamate transporters, and that treatment with n-acetylcysteine or ceftriaxone rescues both expression of xCT and GLT-1, respectively, as well as cocaine-seeking behavior in rats (Knackstedt et al., 2010). Neurons increase the number of dendritic spines upon reinstatement of cocaine, which may be regulated by astrocytes (Hakim et al., 2014; Kalivas, 2009). Together these findings suggest a relationship between glutamate homeostasis, ethanol, and astrocytes.

1.5. Limitations of Current Studies

Behavioral studies in flies and mice provide evidence that glia and neurons communicate and are crucial for regulation of complex behaviors, however the exact method of interaction and role of glia in synaptic plasticity remains to be understood. Recent research has demonstrated novel and complex interactions between astrocytes and neurons to regulate neuronal properties, yet astrocyte function remains largely unknown in both flies and mammals (Perea et al., 2009; Santello et al., 2012). Furthermore, the tools available are lacking, as even though there are hundreds of differentially expressing Gal4 lines are available in flies, there exists a need for both more spatially restricted, and better characterized lines (Rodan and Rothenfluh, 2010).

The mechanisms underlying ethanol tolerance are remarkably poorly understood. As this phenotype lends itself to the formation of AUDs as well as the physiological changes that occur with prolonged alcohol consumption, it is worthy of attention. As a

simple form of change in central nervous system function, ethanol tolerance lends itself nicely to detailed molecular dissection. Further studies can explore how molecular mechanisms of ethanol tolerance apply to the more complex changes accompanying the development of alcoholism, and AUDs. As no drug therapies for AUDs presently target neurons, astrocytes are an appealing target for drug therapies. Furthermore, the use of Drosophila makes it inexpensive to explore astrocyte function with respect to the action of ethanol, potentially facilitating the development of new drug targets. Further research needs to be done with ethanol tolerance and the subsequent changes in gene expression, as this is ultimately what facilitates AUDs.

1.6. Research Aims

The overall goal of this research project is to study the role of glia in ethanol behaviors. This will be achieved through two research aims: 1) characterize how glutamate homeostasis in astrocytes regulates ethanol tolerance; and 2) characterize the blood brain barrier glia's role in ethanol-dependent behaviors.

CHAPTER 2: MATERIALS AND METHODS

2.1 Strains and Culturing Conditions

All strains were outcrossed for at least five generations to the Berlin genetic background. Flies were raised on standard food containing agar (1.2% w/v), cornmeal (6.75% w/v), molasses (9% v/v), and yeast (1.7% w/v) at 25°C and 70% humidity, unless otherwise indicated. Drosophila strains (stock number) were from Bloomington Drosophila Stock Center: *Akap200*^{EY4645} (15759), *UAS-Akap200.IR* (35651), *repo-Gal4* (7415), *nrv2-Gal4* (6800), *Act5C-Gal4* (4414), *UAS-Itp-r83A.IR* (25937), *UAS-Ca-p60A.IR* (25928), *UAS-Pka-R2.IR* (27680), *UAS-Lifeact-GFP* (58718), *UAS-MCFO* (64085); Drosophila Genetic Resource Center: *Akap200*^{NP511} (103626), *Akap200*^{NP609} (103674), *Akap200*^{NP6271} (105177); Vienna Drosophila Resource Center: *UAS-Akap.IR* (v5646), *UAS-CaM.IR* (v28242); Richard Mann: *Akap200*^{PBss}; Roland Bainton: *Indy-Gal4*, *SPG-Gal4*; Marc Freeman: *alrm-Gal4*, *mz709-Gal4*; Paul Garrity: *UAS-TrpA1*; Bing Ye: *UAS-CD2mCherry*; Ulrike Heberlein: *UAS-Pka-C* and *UAS-Pka-C**. *UAS-Akap200* transgenes in the pUAST vector were created by Zhuhao Wu in the laboratory of Alex Kolodkin. The amino acid changes were, in Akap200L isoform PA: *UAS-Akap200.NM* (non-myristoylated mutation): G-2-A, *UAS-Akap200.DN* (non-phosphorylated mutation): S-132/135/137-A, *UAS-Akap200.CA* (pseudo-phosphorylated mutation): S-132/135/137-D.

2.2 Molecular Biology

RNA was extracted from male heads, DNase treated, and reverse-transcribed using MultiScribe™ (Applied Biosystems). Quantitative PCR reactions were done using the Taqman Gene Expression system (Applied Biosystems) and custom designed primers on a StepOnePlus machine (Applied Biosystems). C_t values were normalized to *RpL32*, expression was calculated using the $\Delta\Delta C_t$ method, and the mean of three independent biological replicates was calculated.

2.3 Behavioral Analysis

Ethanol vapor and humidified air were produced as previously described (Wolf et al., 2002). The experimenter was blinded to genotype for all behavioral tests. Groups of 20 male flies were acclimated to a stream of humidified air for 5 min in the booz-o-mat and then exposed to a continuous stream of ethanol vapor. The flies were filmed for locomotion, or they were counted separately for loss of the righting reflex to measure sedation. Sedation sensitivity was calculated as the time to 50% sedation for each group. Sedation tolerance was measured as the time to 50% sedation for exposure 2 minus exposure 1. Locomotion was measured as previously described (Wolf et al., 2002). For behavioral experiments performed at an elevated temperature, I designed a miniaturized booz-o-mat that mounted atop a Peltier thermal controller (IC20, Torrey Pines Scientific). Control and experimental groups were tested side-by-side and across multiple days to account for variation in behavior.

The capillary feeding assay (CAFE) was used to determine ethanol preference, as previously described (Devineni and Heberlein, 2009). Groups of eight adult males were collected 3-4 days after eclosion and allowed to recover from CO_2 for one day. They were then exposed to either 55% ethanol vapor/air mixture or 100% humidified air alone for 20 min. After 16 h recovery, flies were placed into the CAFE chamber, which consists of empty vials with capillary tubes containing liquid food with or without 15% ethanol, embedded in the vial plug. Preference index was measured as the volume of food consumed from the ethanol capillaries minus that consumed from the no-ethanol capillaries over the total volume consumed, corrected for evaporation by measuring the volume lost in vials with no flies. Bitter taste avoidance was measured by presenting flies with a choice of 1.25% agarose containing either 50 mM sucrose (S) or 100 mM sucrose and 1 mM quinine (SQ). Groups of approximately 20 male flies were food deprived on water for 14 h, placed in a 40x90x10 mm clear acrylic arena, and 150 uL S and SQ dots were then placed in apposition at the center of the arena. The number of flies on each dot was counted at 120 min. Avoidance was calculated as (SQ – S)/(SQ + S) such that complete avoidance of bitter gives a value of -1.

Ethanol absorption was measured by exposing groups of 25 flies to either ethanol vapor (40%) or humidified air for 15 minutes. Flies were frozen immediately on dry ice and the ethanol concentration in whole fly homogenates was measured with an alcohol dehydrogenase-based spectrophotometric assay (Diagnostic Chemicals, Ltd., Charlottetown, PE, Canada).

Negative Geotaxis assay was used to determine neurological deficits, as described previously (Barone and Bohmann, 2013). Groups of ten male flies were placed into an empty fly vial that was taped to another one, creating a sealed chamber. Vials were tapped on the benchtop and the number of flies who climbed to or past 8cm in 10seconds were recorded. This was repeated for a total of 10 times, with 30sec of rest in between each trial.

2.4 Immunohistochemistry

Whole flies were prefixed in 4% paraformaldehyde (PF), 1xPBS and 0.05% Triton-X 100 for 20 min on ice to help maintain barrier morphology. Brains were dissected in 1xPBS and 0.05% Triton-X 100 (0.05% PBT), fixed (2% PF in PBT) overnight at 4°C or 1 hr at room temperature. Brains were washed 5x 10 min in 0.1% PBT, blocked 1 hr in 0.1% PBT with 0.5% BSA and 5% normal goat serum and then incubated with primary antibodies overnight at 4°C. Brains were washed, blocked, and incubated with secondary antibodies overnight at 4°C. Brains were washed and mounted on glass slides with Vectashield (Vector Laboratories). Squares of double-sided tape served as narrow spacers such that the brains were partially flattened, facilitating imaging of the brain surface. Primary antibodies used were mouse anti-CD2 (1:500, Serotec OX-34), rabbit anti-GFP (1:1,000, Invitrogen A6455), rabbit anti-dsRed (1:500, Clontech 632496), rat anti-FLAG (1:200, Novus Biotech NBP1-06712), rabbit anti-HA (1:300, Cell Signaling 3724S), and mouse anti-discs large (1:100, DSHB 4F3). MultiColor

FlpOut (MCFO) flies were given a 10-15 min 37°C heat shock and then treated and dissected 2 days later (Nern et al., 2015).

2.5 Dye Injection

Male flies were pressure injected in the medial thorax region with approximately 100 nL of 45 mM 10 kDa Texas Red dextran (Sigma-Aldrich) and 0.2 mM BODIPY FL prazosin (Life Technologies), and allowed to recover for 3 hr (Mayer et al., 2009). Positive control mutants were used to confirm our ability to detect increased permeability of the physical (*moody*^{c17}) and chemical (*mdr65*^{PEx8}) barrier (Bainton et al., 2005; Mayer et al., 2009).

2.6 Statistical Analysis

Statistical analysis was performed with GraphPad Prism v6.0. Typically, one-way ANOVA was used followed with Tukey's multiple comparison test. If the data was not distributed normally (Brown-Forsythe test), we instead used the Kruskal-Wallis test followed with Dunn's multiple comparisons test. t-tests were two-tailed. Sample sizes were chosen based on prior experience with each experimental paradigm. All graphs show the mean and the standard error of the mean.

CHAPTER 3: EXCITOTOXICITY AND ETHANOL TOLERANCE

3.1 Identification of a Glial-specific Gene Involved in Ethanol Response

To identify genes which have changes in expression following ethanol exposure, Kong et al. exposed flies to ethanol or air for 30 minutes and allowed them to recover for no longer than four hours (Kong et al., 2010b). RNA was extracted and analyzed through microarrays. 1,280 genes were found from microarray analysis to have expression changes following ethanol exposure; from these results, 76 mutants were found to be available for screening. Mutant flies were screened with behavioral assays for ethanol tolerance and from this screen 32 genes were identified as either increasing or decreasing tolerance. Of these 32 genes, Akap200, originally thought to be a glial-specific gene, was further explored (**Figure 1**) (Freeman et al., 2003). Characterization of Akap200 is detailed in Chapter 4. Because Akap200 implicated glia in ethanol behavioral plasticity, I performed a broad-based assessment of glial functions that may be important for ethanol tolerance development.

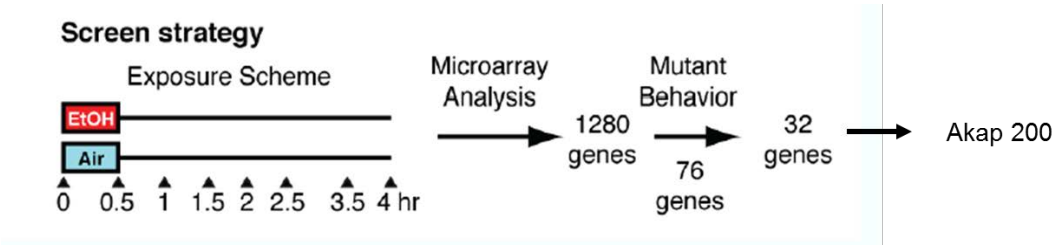


Figure 1: Scheme used to identify candidate genes involved in ethanol exposure.

3.2 Screen for Glial Role in Ethanol Behaviors

My initial screen used a pan-glial driver and the functional manipulations listed in **Table 1**. Expressing these tools pan-glially either resulted in organismal lethality, extreme ethanol sensitivity that is diagnostic for general unhealthiness, or in no ethanol tolerance phenotype (data not shown). I reasoned that the glial subtypes may be differently sensitive to specific manipulations, potentially masking important functions. For this reason, a focus on the role of specific glial cell types was undertaken. I focused on astrocytes due to their proximity to synapses and the recent mammalian literature implicating roles in many neurological functions (Barres, 2008; Danbolt, 2001; Min and Nevian, 2012). To investigate the role that astrocytes play in the development of ethanol sensitivity and ethanol tolerance, behavioral analysis was performed manipulating known mammalian astrocyte functions in fly astrocytes.

Using the GAL4/UAS system I genetically manipulated astrocytes (the alrm-Gal4 driver) in adult flies and measure their behavioral response (see **Table 1**). Vesicular recycling was interrupted using Tetanus Toxin (TeTx), which disrupts exocytosis by cleaving n-synaptobrevin, and Shibire GMR (Shi GMR), which creates a temperature sensitive Dynamin that disrupts vesicular uptake. GPCR signaling was interrupted using Pertussis toxin (PTX), this construct prevents GDP to be swapped out for GTP on the inhibitory Gα-subunit thereby disrupting all inhibitory GPCR signaling, also G-αI RNAi was used to specifically target the inhibitory Gα-subunit pathway, and G-γ RNAi was used to specifically target the Gβγ signaling pathways. The calcium signaling pathways were interrupted using Calmodulin (Cam) RNAi, and Cap60A RNAi, a construct that prevents Itp3 from binding to the SERCA pump on the endoplasmic reticulum. Membrane potential was disrupted using the following transgenes: TrpA1, a temperature-sensitive cation channel will depolarize the membrane when brought above the permissive temperature of 26°C; Zydeco, increases expression of the Sodium Calcium Exchanger (NCX) on the plasma membrane; Kir2.1, is an inwardly rectifying voltage-gated potassium channel that will hyperpolarize the membrane; and NaChBac #1, is the ectopic expression of a bacterial sodium channel causing depolarization of the membrane. Finally, glutamate homeostasis was disrupted by decreasing expression of the glutamate transporter EAAT1.

Table 1: Summary of UAS transgenes of known mammalian astrocyte function and their behavioral response to ethanol exposure. NP is for No Phenotype. Two behavioral readouts were measured, Sedation Sensitivity and Sedation Tolerance.

Transgene	**Cellular Function**	**Sensitivity**	**Tolerance**
TeTx	Vesicular Recycling	NP	*Decreased*
Shi GMR	Vesicular Recycling	NP	NP
PTX	GPCR Signaling	*Increased*	*Decreased*
G-γ RNAi	GPCR Signaling	NP	NP
G-αI RNAi	GPCR Signaling	NP	NP
Cam RNAi	Calcium Signaling	NP	*Decreased*
Cap60A RNAi	Calcium Signaling	NP	NP
TrpA1	Membrane Potential	NP	NP
Zydeco	Membrane Potential	*Increased*	NP
Kir2.1	Membrane Potential	NP	NP
NaChBac #1	Membrane Potential	NP	NP
EAAT1 RNAi	Glutamate Homeostasis	*Increased*	*Decreased*

3.3 Glutamate Regulation by Astrocytes is Required for Ethanol Responses

From this screen, it was noted that disruption of glutamate homeostasis, through the reduced expression of the glutamate transporter EAAT1, led to a very pronounced phenotype (**Figure 2**): increased sedation sensitivity (** p= 0.0055-0.0011) and decreased in sedation tolerance (** p= 0.0032 ***p=0.0002).

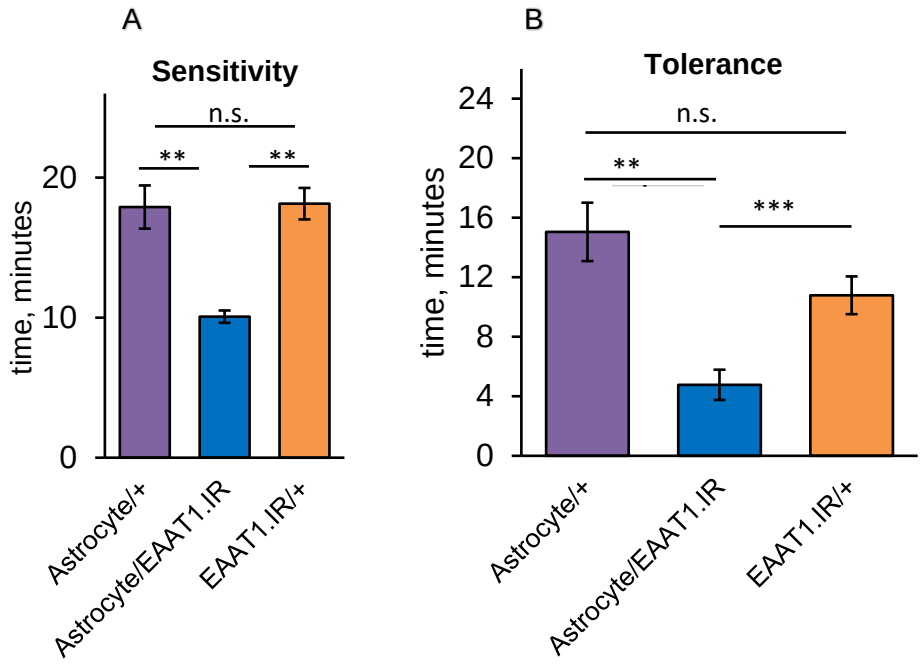


Figure 2: Decreased expression of EAAT1 in astrocytes causes increases sensitivity and decreased tolerance. A) A significant sedation sensitivity phenotype is seen. B) Decreased tolerance is seen in experimental flies (blue bar) compared to controls. One-way ANOVA/Tukey's, n=8-9 groups of flies. **P<0.01, ***P<0.001

To test if the phenotype seen with the RNAi was a result of the lack of transporter, an opposing experiment was performed by ectopically overexpressing EAAT1 in astrocytes. Ectopic overexpression of EAAT1 provided an opposing phenotype, with no sedation sensitivity but increased ethanol tolerance (**Figure 3**). This confirms that the original phenotype with the RNAi was not a result of the gross disruption of the nervous system, but rather a result of the decreased expression of EAAT1. Further, it suggested that either glutamate clearance from the synapse, or a product of glutamate metabolism, may play a central role in setting ethanol tolerance susceptibility.

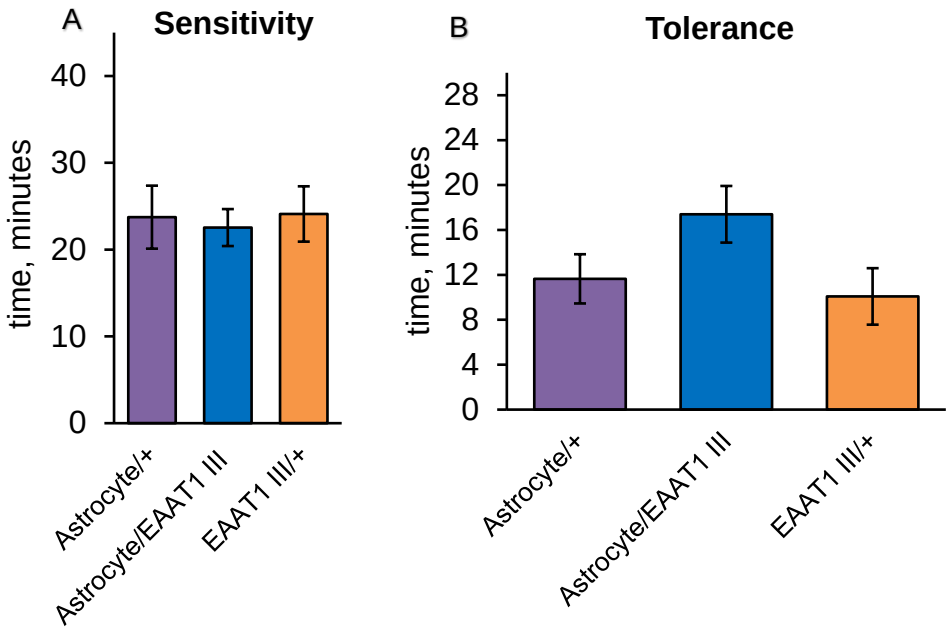


Figure 3: Overexpression of EAAT in astrocytes provides no sensitivity or tolerance phenotype. A) No sedation sensitivity phenotype is seen. B) No significant tolerance phenotype is seen in experimental flies (blue bar) compared to controls. One-way ANOVA, n=11 groups of flies.

3.4. Dysregulation of Glutamate Homeostasis Causes Signs of Neurodegeneration

Climbing ability is a measure of overall nervous system function: excitotoxicity from reduced glutamate clearance, and other neurodegenerative conditions reduce climbing (Barone and Bohmann, 2013). Flies with reduced EAAT1 in astrocytes showed a pronounced climbing defect (**Figure 4**). Concern was made that perhaps the flies were not able to properly right themselves following the spinning of tubes in the Sedation Tolerance assay, thus readings of knock-out flies were merely flies that were stuck versus intoxicated. To confirm that this climbing defect did not compromise the original behavioral phenotype I repeated the original Sedation Tolerance assay without ethanol (data not shown). The results confirmed that despite having an inability to climb, the experimental flies could right themselves without defect, showing that the original behavioral phenotypes are due to ethanol-induced behavior. Furthermore, our climbing assay was performed on newly eclosed flies. Overt signs of neurodegeneration with reduced EAAT1 do not become evident until at least 15 days, which is roughly late middle age for a fly. These data suggested that reducing glutamate clearance and homeostasis by astrocytes may cause pre-neurodegenerative changes to neurons important for ethanol tolerance.

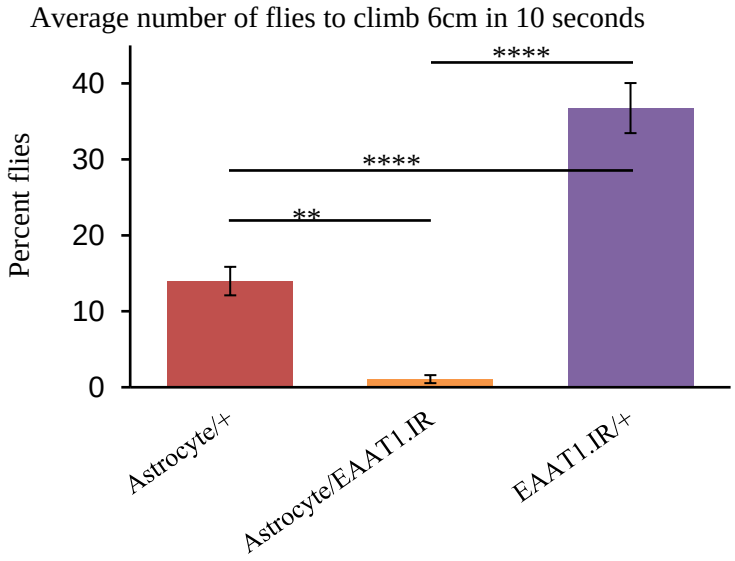


Figure 4: Decreased expression of EAAT1 in astrocytes provides a locomotor defect phenotype. Average results of climbing assay for individual trials and genotypes showing significant climbing defect in experimental flies. One-way ANOVA/Tukey's, n=11 groups of flies. **P<0.01, ****P<0.0001

Another potential explanation for the ethanol tolerance phenotypes with changes in EAAT1 expression levels is that ethanol absorption or metabolism may be affected. An ethanol metabolism assay was conducted to assess if the behavioral phenotype was not due to altered ethanol metabolism (**Figure 5**). Flies were exposed to ethanol or air for 15 minutes and either frozen immediately or allowed to metabolize for 30 minutes and then frozen. Ethanol content was analyzed by measuring ethanol concentration in exposed flies and subtracting the values found in unexposed control flies. No significant differences were seen between experimental and control flies at either the 15 minute or 45 min time points. Thus, experimental flies were not merely containing an increased amount of alcohol and responding accordingly, but were exhibiting a change in behavior due to the lack of functional EAAT1 in the astrocytes.

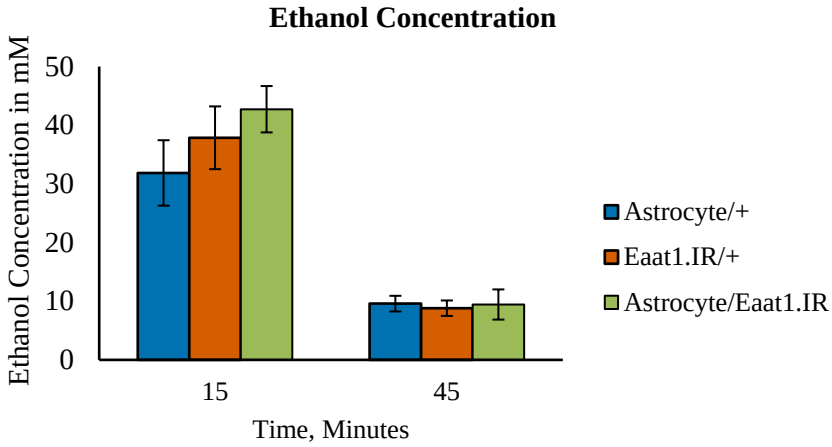


Figure 5: Decreased expression of EAAT1 in astrocytes does not affect ethanol metabolism. Bar graph displaying ethanol metabolism in flies. No visible differences in either initial ethanol absorption (15 minute) or ethanol metabolism over time (45 minutes) across groups and genotypes. One-way ANOVA, n=4 groups of flies.

Excitotoxicity causes a significant decrease in longevity. To test if flies with reduced EAAT1 were short-lived, I assessed viability over a 75 day period. Flies were exposed to either ethanol or air for 45 min and then were cultured under standard conditions to determine how exposure would affect the lifespan of the flies. Our results were consistent with previous findings, with flies expressing decreased levels of EAAT in astrocytes exhibiting decreased lifespan (**Figure 6**) (Rival et al., 2004). Ethanol exposure did not hasten demise or proffer a protective effect, either in the experimental or the controls (**Figure 6**). This data suggests that ethanol does not interact synergistically with glutamate dyshomeostasis to alter the rate of neurodegeneration.

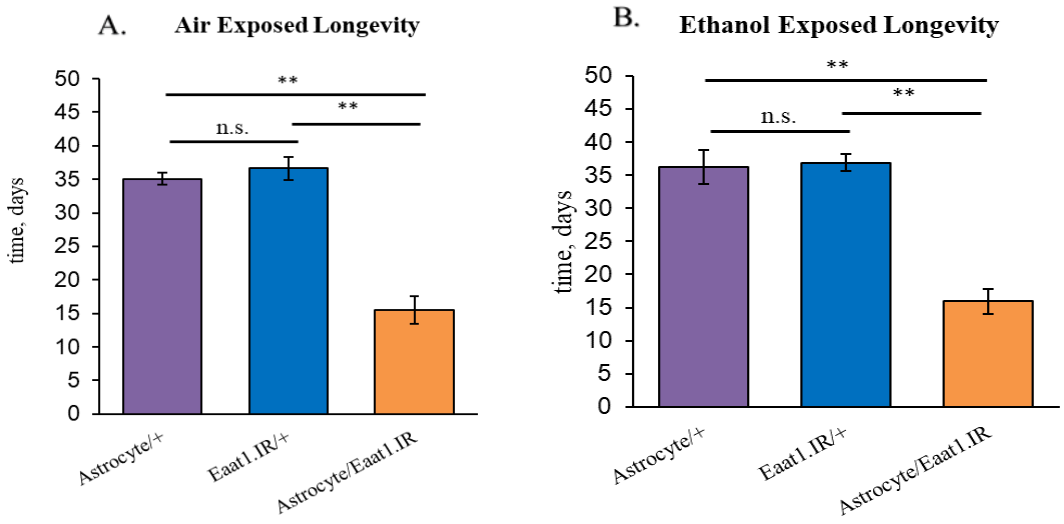


Figure 6: Decreased expression of EAAT1 in astrocytes reduces fly lifespan. A) Flies exposed to air only showed decreased longevity compared to controls. B) Flies exposed to ethanol also showed decreased in longevity compared to controls. One-way ANOVA/Tukey's, n=15 groups of flies. **P<0.01

3.5. Discussion

In summary, disruption of glutamate uptake by fly astrocytes strongly alters ethanol sensitivity and tolerance. In tandem, it degrades behavioral responses by increasing neurodegeneration. Previous studies have unequivocally demonstrated that blocking glutamate uptake by astrocytes in flies results in excitotoxicity from increased glutamate neurotransmission (Aw et al., 2017; Rival et al., 2004, 2006; Verma et al., 2015). The mechanism is thought to be tied to increased production of reactive oxygen species in the overstimulated neurons (Bingol et al., 2014; Cassar et al., 2015; Yu et al., 2011). We were unable to separate excitotoxicity from ethanol behavioral phenotypes. Therefore, either excitotoxicity results in changes in ethanol behavioral responses, or they are separate processes that are difficult to untangle at the present. It is important to note that onset of the neuroanatomical overt behavioral signs of neurodegeneration typically take 10-20 days. Our ethanol tolerance tests are done on younger flies that are a maximum of 5 days old. This may suggest that damage to the nervous system occurs much earlier than previously thought. Alternatively, ethanol tolerance is a separable process. A further weak argument against neurodegeneration as the sole cause of decreased ethanol tolerance: Eaat1 overexpression in astrocytes rescues neurodegeneration in a fly model for tauopathy, whereas I found no effect on ethanol tolerance (Kilian et al., 2017).

Decreasing EAAT1 in astrocytes not only increases extracellular glutamate, but also decreases glutamate availability inside the astrocytes. I also pursued manipulation of astrocyte glutamate metabolism, but these manipulations either had no clear effect on ethanol behavioral responses or caused early lethality (data not shown).

The current state of technology limits our ability to firmly answer whether glutamate homeostasis by astrocytes contributes to ethanol behavioral plasticity. However, our data does indicate that astrocytes function in ethanol behavioral responses. In addition to glutamate, we found that signaling by inhibitory GPCRs in astrocytes is important for tolerance. Astrocytes express GPCRs for almost all neurotransmitters and neuromodulators (Zhang et al., 2014). GPCRs conduct slower neurotransmission, and therefore generally transmit modulatory information to the receiving cells. Our admittedly preliminary data suggests that astrocytes are modified by ongoing neurotransmission that is affected by ethanol, and this may be tied to glutamate homeostasis. The advent of tools to alter the function of just a subset of astrocytes, especially those in direct contact with neurons that promote ethanol behavioral plasticity, will give us much needed specificity and will allow us to readdress the questions raised by our experiments (Engel et al., 2016; Jenett et al., 2012; Urizar et al., 2007).

CHAPTER 4: THE BLOOD BRAIN BARRIER IS PHYSICALLY REMODELLED BY ETHANOL IN AN AKAP200-DEPENDENT MANNER

4.1 Akap200 Mutants Exhibit Decreased Behavioral Plasticity Upon Repeated Ethanol Exposure

To understand if Akap200 functions in ethanol behavioral responses, we first characterized its transcriptional response to ethanol. The *Akap200* locus transcribes seven distinct transcripts that are translated into two major forms of Akap200 protein (**Figure 7a**). Class I transcripts encode Akap200L proteins that contain a protein kinase A (PKA) regulatory subunit type II (RII) binding domain, and Class II transcripts encode Akap200S proteins that lack the RII domain. We confirmed increased *Akap200* expression with ethanol exposure, and found that Class I transcripts were upregulated by ethanol (**Figure 7b**). To ask if *Akap200* functions in ethanol behavioral responses, we characterized new mutations in the gene. Two transposon insertions in the *Akap200* locus, *NP511* and *EY4645*, strongly decreased expression of *Akap200* transcript classes in homozygotes (**Figure 7c**). Two additional independent transposon insertions at different genomic locations also decreased *Akap200* expression (**Figure 7c**). All these

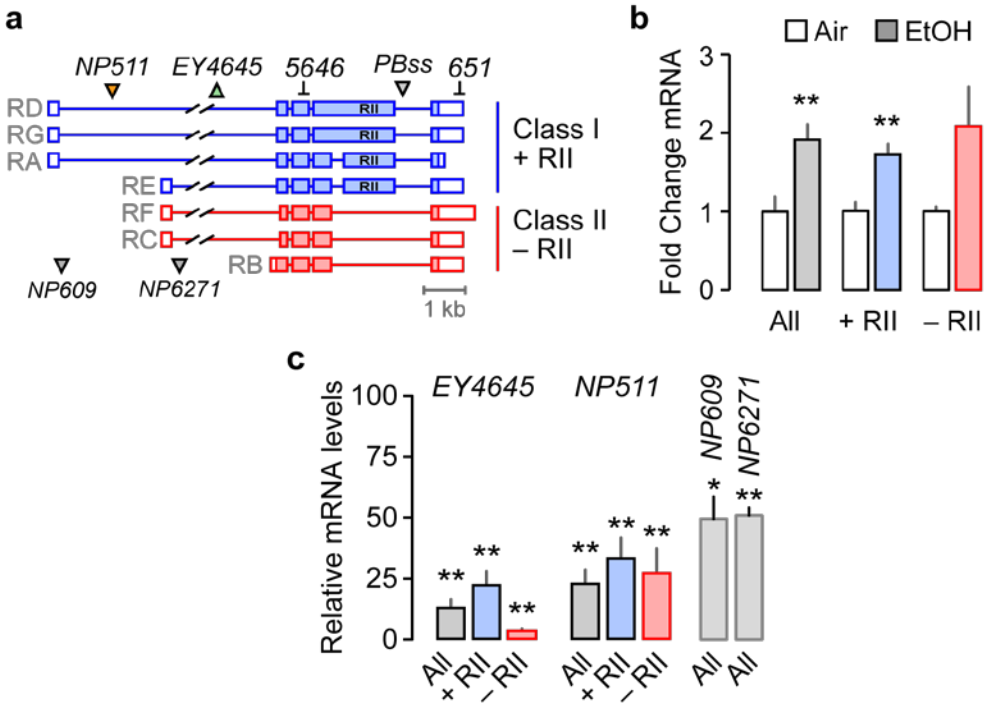


Figure 7. *Akap200* expression levels are increased by ethanol. a. Map of the *Akap200* genomic region depicting the two major transcript classes that encode distinct proteins. Boxes indicate exons and open reading frames are shaded. Triangles indicate transposon insertion sites, and sequences used to generate dsRNA (*5646, 651*) are also indicated. qPCR probesets bridge the final intron for each class. **b**. *Akap200* transcript levels 1 h after exposure to 30' humidified air or 30' ethanol vapor. One way ANOVA/Dunn's, **P<0.01. n=7 biological replicates. **c**. Expression of *Akap200* is reduced in flies homozygous for the indicated transposon insertions. *P<0.05 **P<0.01 one sample t-test. n=5-7 biological replicates.

Akap200 mutants were homozygous viable and sterile. The sterility phenotype is likely due to the role of Akap200 in oogenesis (Jackson and Berg, 2002).

We tested *NP511* and *EY4645* flies for their behavioral response to ethanol using assays that measure sedation sensitivity (**Figure 8a**) and locomotor stimulation (**Figure 8f**). To induce and measure rapid tolerance to ethanol sedation, flies were exposed twice to ethanol vapor, with a 4 h interval between the start of each exposure, allowing for complete ethanol metabolism between exposures. Flies are less sensitive to the sedating effects of ethanol upon the second exposure, and this tolerance is measured as the difference in time to 50% sedation (ST50) between exposures (**Figure 8a**). *EY4645* flies showed increased sensitivity to the sedative effect of acute ethanol exposure (**Figure 8b**), and both *EY4645* and *NP511* showed decreased ethanol tolerance (**Figure 8c**). Flies heterozygous for either mutation were unaffected for ethanol behavioral responses (**Figure 8d,e**), indicating that both *Akap200* mutations are recessive and loss-of-function for ethanol sedation responses. The two additional alleles, *NP609* and *NP6271*, also exhibited decreased ethanol tolerance (**Figure 8c**).

Inebriating ethanol doses stimulate locomotor activity, and this ethanol-induced hyperactivity is sensitized upon a second exposure (**Figure 8f**) (Kong et al., 2010b). A measure of hyperactivity is the total distance travelled between 2 and 25 min of ethanol exposure, and the difference between exposures, ΔDist, is a measure of behavioral plasticity. *NP511* increased and *EY4645* decreased ethanol-induced hyperactivity (**Figure 8g**), whereas both mutations decreased ΔDist (**Figure 8h**). Ethanol absorption was unaffected in the *Akap200* mutant flies (**Figure 8i**).

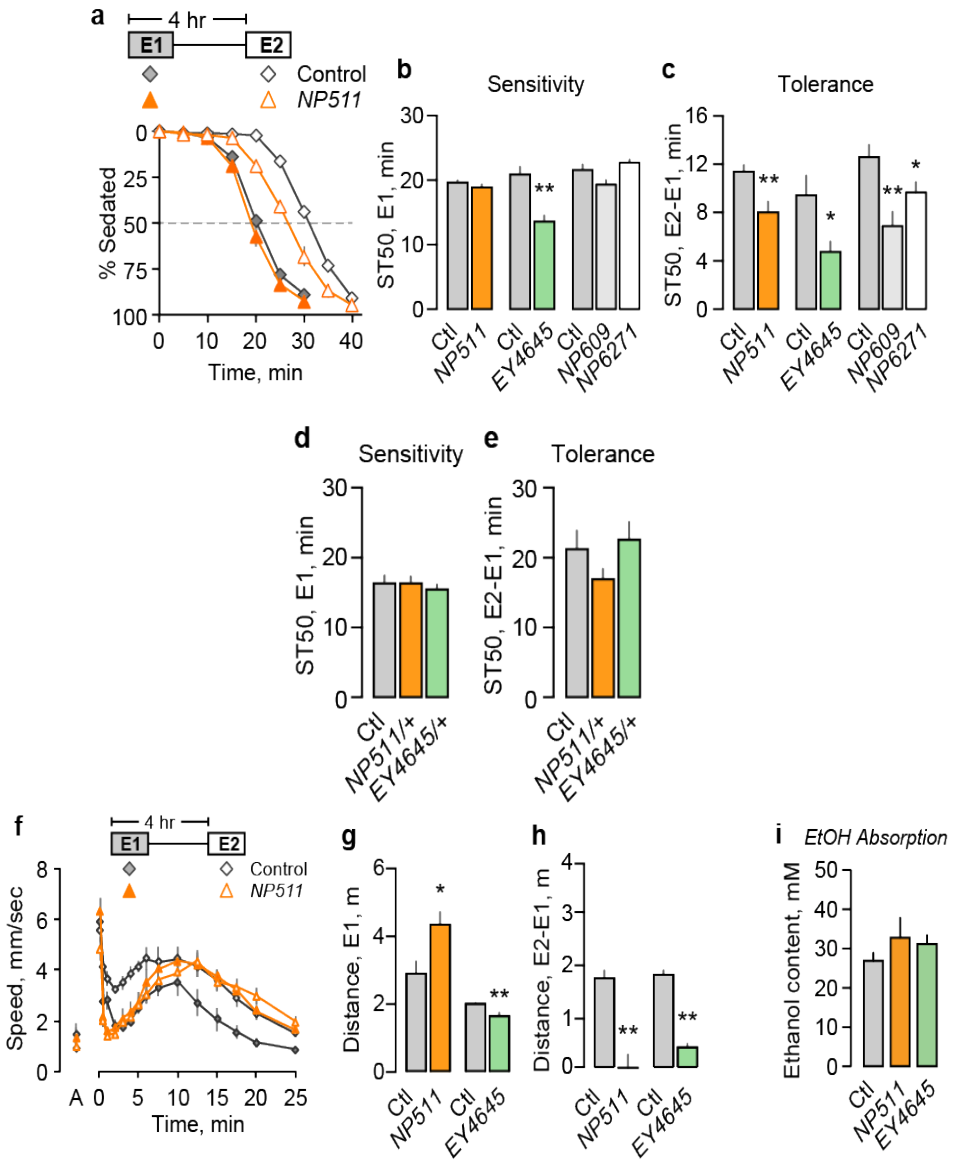


Figure 8. **Ethanol behavioral responses are altered in *Akap200* mutants. a**. Ethanol sedation sensitivity and tolerance time course. Flies are given two identical exposures to continuous ethanol vapor (E1, filled symbols, and E2, open symbols) separated by a rest period. ST50 is the time to 50% sedation within a group of approximately 20 flies. n=9 groups of flies. **b**-**e**. Sedation sensitivity (**b**, **d**) and tolerance (**c**, **e**) for flies homozygous (**b**, **c**) or heterozygous (**d**, **e**) for *NP511*, *EY4645*, *NP609*, or *NP6271*. Homozygous: one-way ANOVA, n=7-12 groups of flies. Heterozygous: one-way ANOVA, n=6-20 groups of flies. **f**. Ethanol-induced locomotor activity using the exposure scheme and genotypes described in **a**. 'A' indicates the locomotor speed of flies in a stream of humidified air just prior to continuous ethanol exposure. **g**. Distance travelled from 2-25 min ethanol exposure. *P<0.05 **P<0.01 t-test. n=6-7 groups of flies. **h**. Distance travelled, E2-E1. **P<0.01 t-test. n=6-7 groups of flies. **i**. Internal ethanol concentration in flies exposed to ethanol vapor. n=3-4 groups of flies. 'Ctl' is the Berlin genetic background strain.

Ethanol preference is a distinct measure of drug-induced behavioral plasticity (Devineni and Heberlein, 2009; Devineni et al., 2011; Ja et al., 2007). Drug naïve flies equally prefer food and food+ethanol, whereas pre-exposure to an inebriating dose of ethanol vapor induces preference for food+ethanol (**Figure 9a,b**) (Peru Y Colón de Portugal et al., 2014). Interestingly, *Akap200* mutant flies preferred food+ethanol even without the priming pre-exposure (**Figure 9b**). This precocious ethanol preference could be due to an inability to detect aversive tastants such as ethanol. However, *Akap200* mutant flies given a choice between highly sweet but bitter and less sweet foods chose the less sweet option (**Figure 9c**). These data show that *Akap200* affects ethanol sensitivity and promotes behavioral plasticity (tolerance, ΔDist, and preference) upon repeated or chronic ethanol exposure.

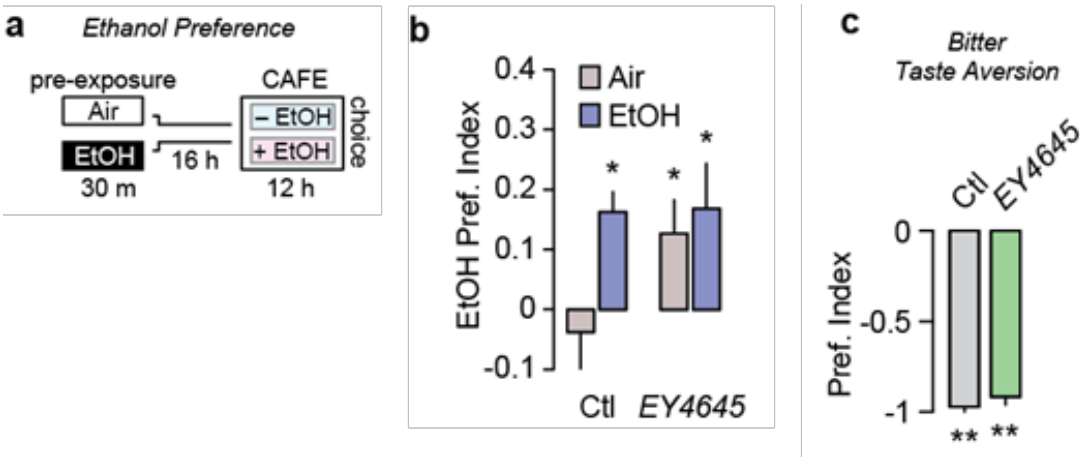


Figure 9. *Akap200* mutants prefer ethanol without a priming ethanol exposure. **a**. Ethanol preference measured in the CAFÉ assay. Flies were pre-exposed to ethanol vapor (EtOH) or humidified air (Air), allowed to recover for 16 hr, and placed in the CAFÉ assay overnight to measure preference for ethanol consumption. **b**. While *Akap200* mutants developed ethanol preference, they exhibited preference without prior ethanol experience (lower panel). *P<0.05, one sample t-test compared to 0. n=19-22 groups of flies. **c**. Bitter taste aversion in flies given the choice of sucrose with and without quinine. A value of '-1' indicates complete aversion. *P<0.01, one-sample t-test compared to 0. n=5-9. 'Ctl' is the Berlin genetic background strain.

4.2 *Akap200* Expression in the Adult Nervous System

Akap200 is expressed almost exclusively in the nervous system during embryonic development (Bonin and Mann, 2004; Freeman et al., 2003). We used the GFP protein trap *PBss* (**Figure 7a**) to assess Akap200 distribution in the adult nervous system. In *Akap200*^{PBss}, *GFP*-encoding sequences are predicted to splice to the *Akap200* open reading frame upstream of the final coding exon, and downstream of all characterized functional domains. We confirmed the presence of Akap200-GFP fusion proteins in *Akap200*^{PBss} fly heads by western analysis (**Figure 10a**), detecting both Akap200L-GFP and Akap200S-GFP. Akap200-GFP appeared cytoplasmic and was widely distributed in the brain (**Figure 10b**). Particularly strong expression surrounding neuronal nuclei in the cortex suggested expression in cortex glia. Ensheathing glia that surround neuropils like the mushroom body kenyon cells and the antennal lobe glomeruli also appeared to be labeled. Diffuse expression was evident throughout the synaptic neuropil. We used the *Gal4*/*UAS* binary expression system to express two *Akap200* dsRNAs, *651* and *5646*, that target distinct regions of *Akap200* that are common to all isoforms (**Figure 7a**). When expressed ubiquitously, both dsRNAs decreased *Akap200* expression to barely detectable levels (**Figure 10e**). *Akap200* RNAi restricted to all neurons reduced Akap200-GFP throughout the adult brain, with the greatest reduction in the synaptic neuropil, and made ensheathing glial Akap200-GFP expression more visible (**Figure 10c,c'**). Glial-specific *Akap200* RNAi also reduced Akap200-GFP expression, especially in the cortex (**Figure 10d,d'**). Thus, Akap200 is present in both neurons and glia in the adult brain, and Akap200 expression is widespread.

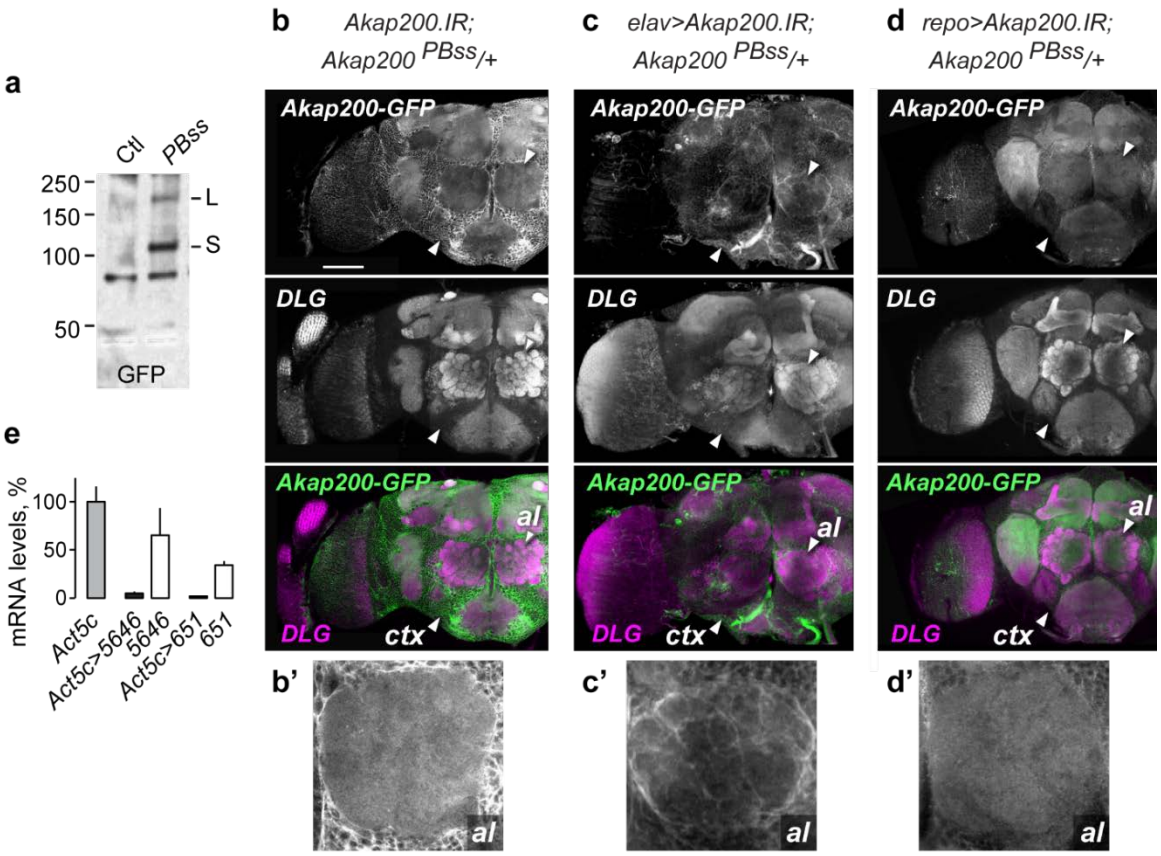
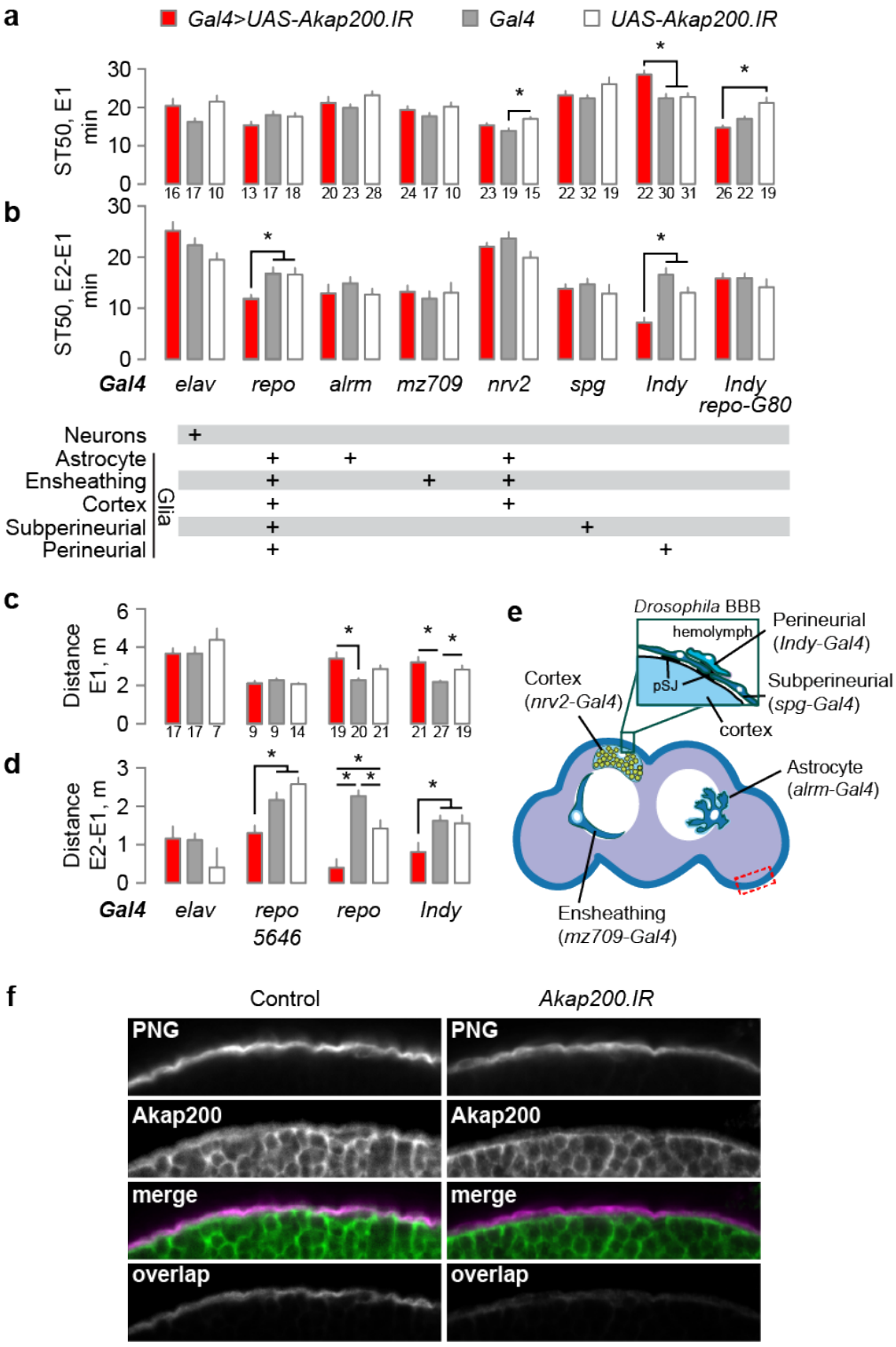


Figure 10. **Akap200 expression in the adult brain. a**. The Akap200-PBss protein trap produces endogenous GFP-tagged Akap200L and Akap200S (Akap200-GFP). Western analysis of whole head protein extracts from genetic background control and *Akap200^{PBss}* flies, probed with GFP antibodies. Molecular weights are in kDa. **b**. Akap200-GFP is expressed broadly in the adult fly brain. Confocal image of a *w,UAS-Akap200.IR/+;Akap200^{PBss}/+* control brain (without a GAL4 driver transgene) labeled with antibodies to GFP (green) and DLG (magenta) to broadly label brain synapses. ctx: cortex region containing glia and neuronal cell bodies; al: antennal lobe. Scale bar: 50 µm. **b'**. Enlarged view of Akap200-GFP in the antennal lobe. **c**. *Akap200* RNAi targeted to all neurons globally reduced Akap200-GFP expression, especially in the synaptic neuropil. **c**'. Reduced synaptic region staining reveals Akap200-positive ensheathing glia. Genotype: *w,elav(c155)-Gal4,UAS-Akap200.IR/+;Akap200^{PBss}/+*. **d**. *Akap200* RNAi targeted to all glia also reduced Akap200-GFP expression. **d'**. Only synaptic neuropil Akap200-GFP staining is evident in the antennal lobe. Genotype: *w,UAS-Akap200.IR/+;Akap200^{PBss}/+;repo-Gal4/+*. **e**. *Akap200* transcript levels are reduced in fly heads when *Akap200* RNAi is expressed ubiquitously with *Act5c-Gal4*.

4.3 *Akap200* is Required in the Outermost Layer of the Blood-brain Barrier for Ethanol Responses

We next asked if *Akap200* was required in the nervous system for ethanol responses. *Akap200* RNAi in all neurons (*elav-GAL4*) did not alter ethanol sedation sensitivity or tolerance, whereas *Akap200* RNAi in all glia (*repo-GAL4*) reduced ethanol tolerance (**Figure 11a,b**). Drosophila possess diverse types of glia that perform specific functions (**Figure 11e**), and so we used a panel of transgenes that express Gal4 in specific glial classes to reduce *Akap200* expression (**Figure 11b**, lower table). *Akap200* RNAi specifically in perineurial glia (using *Indy-Gal4*) decreased ethanol sedation sensitivity and sedation tolerance (**Figure 11a,b**). *Indy-Gal4* is highly specific to the perineurial glia in the adult nervous system (**Supplementary Figure 1**), however it may be expressed in other tissues in the animal (DeSalvo et al., 2011a). To test this, we introduced *repo-Gal80*, a transgene that expresses the GAL4 inhibitor GAL80 specifically in all glia, and we found that it blocked the behavioral effects of *Akap200* RNAi driven by *Indy-Gal4*, indicating that Akap200 function for ethanol tolerance is specific to perineurial glia (**Figure 11a,b**). Like ethanol sedation tolerance, ΔDist was decreased when *Akap200* expression was reduced in all glia and specifically in perineurial glia (**Figure 11c,d**). These findings indicate that *Akap200* promotes ethanol behavioral responses through its actions in the perineurial glia that form the outermost cellular layer of the Drosophila blood-brain barrier. Higher magnification images revealed Akap200-GFP expression in perineurial glia (**Figure 11f**). Perineurial-specific *Akap200* RNAi decreased Akap200-GFP expression specifically in these cells, and the cell layer appeared to be intact (**Figure 11f**). There is limited understanding of the function of the perineurial glia (Seabrooke and O'Donnell, 2013; Volkenhoff et al., 2015).

Figure 11. ***Akap200* is required in the perineurial glia for ethanol responses**. **a**,**b**. Ethanol sedation sensitivity (**a**) and tolerance (**b**) when expressing *Akap200* dsRNA (*UAS-Akap200.IR*) in neuronal and glial cells with the *Gal4* strains indicated below the graphs. Tissue type expression is listed for each *Gal4* strain below the graphs. *repo-G80* expresses the GAL4 inhibitor GAL80 specifically in all glial cells. One way ANOVA/Tukey's, the number of groups tested is indicated below each bar. **c**,**d**. Ethanol induced locomotor activity in ethanol naïve (**c**) and ethanol pre-exposed (**d**) flies. One way ANOVA/Tukey's, the number of groups tested is indicated below each bar. All tests were with *UAS-Akap200.IR* 651 except where indicated. **e**. Diagram depicting the location of the five types of glia in flies. Diagram adapted with permission from Dr. Margaret Ho (Ou et al., 2016). **f**. Akap200 is expressed in the perineurial glia, labeled with the *Indy-Gal4* transgene. "Overlap" show pixels that are common between labels. Genotypes are Control: *w*;*Akap200*^{PBss}/*UAS-CD2mCherry*;*Indy-Gal4*/+, and *Akap200.IR*: *w*,*UAS-Akap200.IR*;*Akap200*^{PBss}/*UAS-CD2mCherry*;*Indy-Gal4*/+. Labeled with dsRed (magenta, to detect CD2mCherry in the perineurial glia) and GFP (green, for Akap200-GFP) antibodies. Area imaged is depicted by a dashed rectangle in panel **e**. Scale bar: 25 μm.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alrm
mz709
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spg
Indy
Indy repo-G80
Neurons
Glia
Astrocyte
Ensheathing
Cortex
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Astrocyte (alrm-Gal4)
Ensheathing (mz709-Gal4)
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4.4 Ethanol Alters Perineurial Glial Morphology in an *Akap200*-dependent Manner

The perineurial layer of the blood-brain barrier is composed of elongated cells that tile the entire surface of the adult brain (Awasaki et al., 2008; Kremer et al., 2017; Stork et al., 2008). Labeling the perineurial glia with plasma membrane bound GFP reveals their tiled organization on the front surface of the brain (**Figure 12a**, Air panel, and **Supplementary Figure 2**). *Akap200* RNAi in the perineurial glia did not affect perineurial morphology, indicating that their development and overall structure is unaffected by loss of *Akap200* (**Figure 12b**, Air panel). Exposure of awake and behaving flies to an ethanol dose that induces tolerance resulted in a marked change in perineurial glia membrane topology (**Figure 12b**, Ethanol panel), appearing more disorganized and masking the tiled appearance. We used Multicolor FlpOut to assess the morphology of individual cells (Nern et al., 2015). Whereas the spatial arrangement of perineurial cells appeared unaffected by ethanol, the plasma membranes appeared less uniform (**Supplementary Figure 2a,b**). Because Akap200 binds actin, we expressed the Lifeact-GFP actin-binding protein in perineurial glia, which was stochastically expressed in a subset of perineurial cells. The actin cytoskeleton in untreated perineurial cells appears to form a lattice-like structure **(Supplementary Figure 2c**). Ethanol exposure disrupted the lattice, and resulted in the accumulation of discrete actin blobs **(Supplementary Figure 2d**).

Surprisingly, the altered appearance of the perineurial glia persisted at least 24 h after recovery from the single ethanol exposure, a time when tolerance has mostly dissipated (**Figure 12a**, 24 hr rest panel) (Scholz et al., 2000). In flies with *Akap200* expression reduced specifically in the perineurial glia, ethanol exposure affected perineurial glia membrane topology less severely or in a delayed manner (**Figure 12b**, lower panels). These data indicate that ethanol induces morphological changes in the perineurial glia through an Akap200-dependent mechanism.

Ethanol may promote tolerance by structurally or functionally incapacitating the perineurial glia. We tested the extreme form of this scenario by killing the perineurial layer specifically in adults. Flies of the genotype *tub-Gal80^{ts},Indy-Gal4>UAS-rpr,UAS-hid* will express pro-apoptotic Rpr and Hid in the perineurial glia at 29°C but not at 18°C. Flies raised at 18°C were viable and outwardly normal. When shifted to 29°C as adults nearly all experimental flies died within 3 days, whereas temperature controls maintained at 18°C and genetic controls lacking *UAS-rpr* and *UAS-hid* shifted to 29°C remained alive. Furthermore, ethanol exposed wild-type flies showed no decreased viability for at least a week after exposure (not shown). Therefore, the adult perineurial glia are essential for viability, consistent with their known essential role in transporting circulating sugars into the brain, and they retain their vital functions following ethanol exposure (Volkenhoff et al., 2015).

The subperineurial glia make extensive physical contact with the perineurial glia and they form both the physical and chemical barrier in Drosophila (Hindle and Bainton,

2014). Ethanol exposure or loss of *Akap200* could affect behavior by changing barrier permeability, potentially altering the molecular composition of the brain extracellular fluid. Previous work showed that disruption of the subperineurial physical barrier decreased ethanol sensitivity (Bainton et al., 2005). We asked if either ethanol or the loss of *Akap200* disrupted these protective functions of the barrier. Dye-coupled large molecular weight dextrans injected into the hemolymph are physically excluded from the brain by subperineurial septate junctions, and we observed that the injected dye accumulated at the perineurial glia (**Figure 12d**). Similarly, the drug prazosin is chemically excluded from the brain by subperineurial transporter proteins; we confirmed that BODIPY-prazosin accumulated at the blood-brain barrier along with dextran (**Figure 12e**) (Mayer et al., 2009). Neither loss of *Akap200* nor ethanol exposure resulted in increased penetration of either molecule into the brain (**Figure 12e**). Tests with gene mutants known to disrupt the physical (*moody*) and chemical (*mdr65*) barrier confirmed our ability to detect barrier defects (not shown). Therefore, the classical barrier functions of the blood-brain barrier appear to be intact following ethanol treatment and with loss of *Akap200*, suggesting that *Akap200* serves a different role in the perineurial glia.

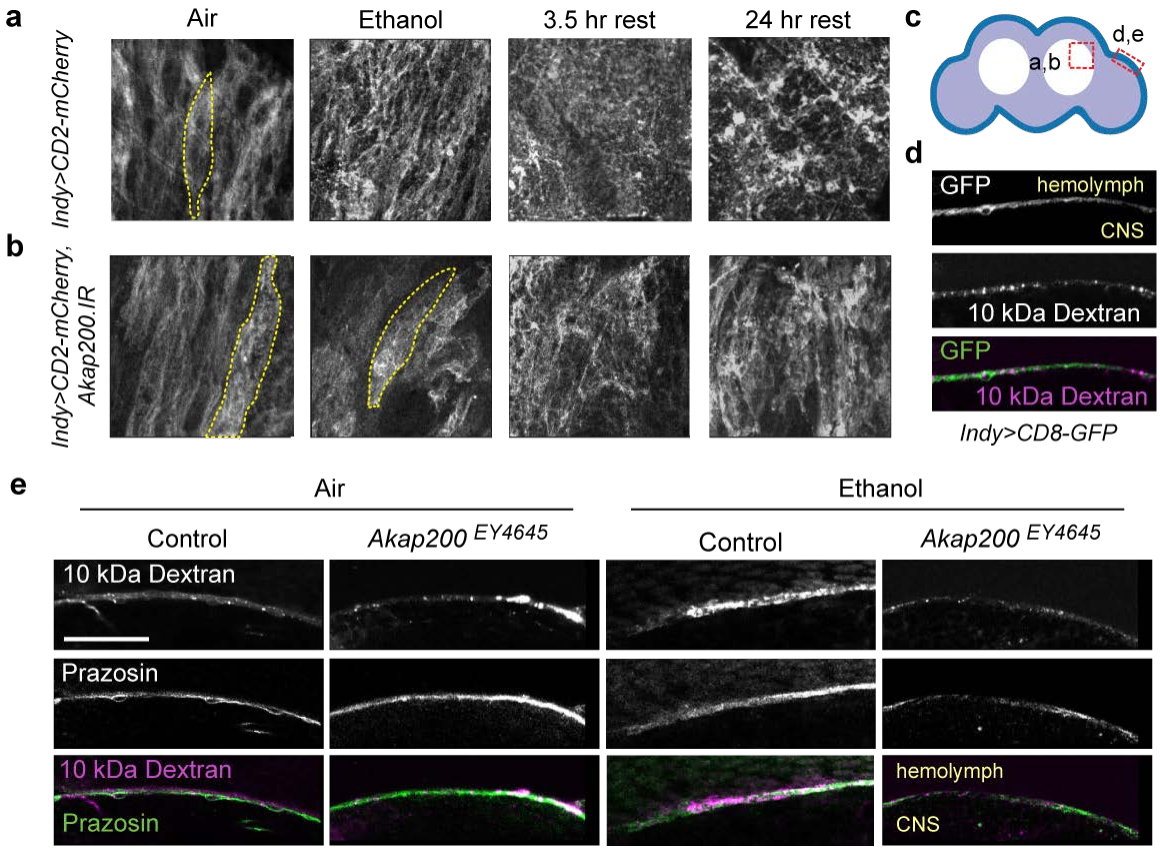


Figure 12. **Ethanol causes a morphological change of the perineurial glia that depends on perineurial glia-expressed Akap200. a.** Front surface image of the central brain perineurial glia expressing plasma membrane-bound mCherry, revealing the columnar distribution of the elongated perineurial glia cells in ethanol naïve flies (Air). An individual cell is outlined. Membrane topology became more complex immediately following exposure to a just sedating dose of ethanol (Ethanol). This effect persisted at least 24 h post-ethanol recovery (rest). **b.** Ethanol effects on perineurial glia topology were delayed and less dramatic when Akap200 expression was reduced in the perineurial glia. **c**. Brain areas imaged in this figure. **d.** High molecular weight dye (10 kDa Texas Red dextran) injected into the hemolymph accumulated at the perineurial glia layer (*Indy-Gal4*/+;*UAS-CD8:GFP*/+) of the blood-brain barrier, and did not penetrate into the central nervous system (CNS). **e**. The physical and chemical exclusion properties of the blood-brain barrier were unaffected by ethanol exposure or lack of *Akap200*. 10 kDa Texas Red dextran (physical, septate junctions, magenta) and BODIPY-prazosin (chemical, green) co-injected into the hemolymph were excluded from the CNS. Micrographs depict 3 µm frontal sections midway through the brain, at the surface of the optic lobe. Scale bar: 25 µm.

4.5 Akap200 Overexpression in the Perineurial Glia Decreases Ethanol Tolerance

To determine if Akap200 regulates ethanol responses in a dose sensitive manner, we overexpressed Akap200 in the perineurial glia. Akap200L (Long) overexpression increased ethanol sensitivity and decreased ethanol tolerance, similar in effect to the *EY4645* loss-of-function mutation, whereas overexpression of Akap200S (Short) had no effect (**Figure 13a,b**). Because Akap200L contains the PKA-RII binding domain, this suggests that spatial coordination of PKA by Akap200 in the perineurial glia can affect ethanol behavioral responses. This overexpression phenotype gave us an opportunity to test the importance of each Akap200 domain (Rossi et al., 1999). PKC phosphorylation sites in the N-terminal positively charged domain were made either nonphosphorylatable (S->A) or psuedophosphorylated (S->D), and the myristoylation consensus sequence was separately mutated. Both psuedophosphorylation and blocking myristoylation eliminated the effects of overexpression on ethanol sensitivity and tolerance (**Figure 13a,b**). Because PKC phosphorylation dissociates calmodulin and actin from Akap200, these data suggested that Akap200 membrane localization and its interactions with PKC, calmodulin, or actin may be important for its role in ethanol behavioral responses (Rossi et al., 1999).

To test if increased Akap200L expression resulted in a dominant negative effect, interfering with normal Akap200 function, or a neomorphic effect, we overexpressed wild-type Akap200L in flies heterozygous for the strong loss-of-function allele *EY4645* to lower the dose of endogenous Akap200. Decreasing the dose of Akap200 had no effect on Akap200 overexpression behavioral phenotypes (**Figure 13c,d**), suggesting that overexpression conferred a new function to Akap200 that impacts ethanol behavioral responses. Alternatively, Akap200 levels may need to be kept in a tightly controlled optimal range.

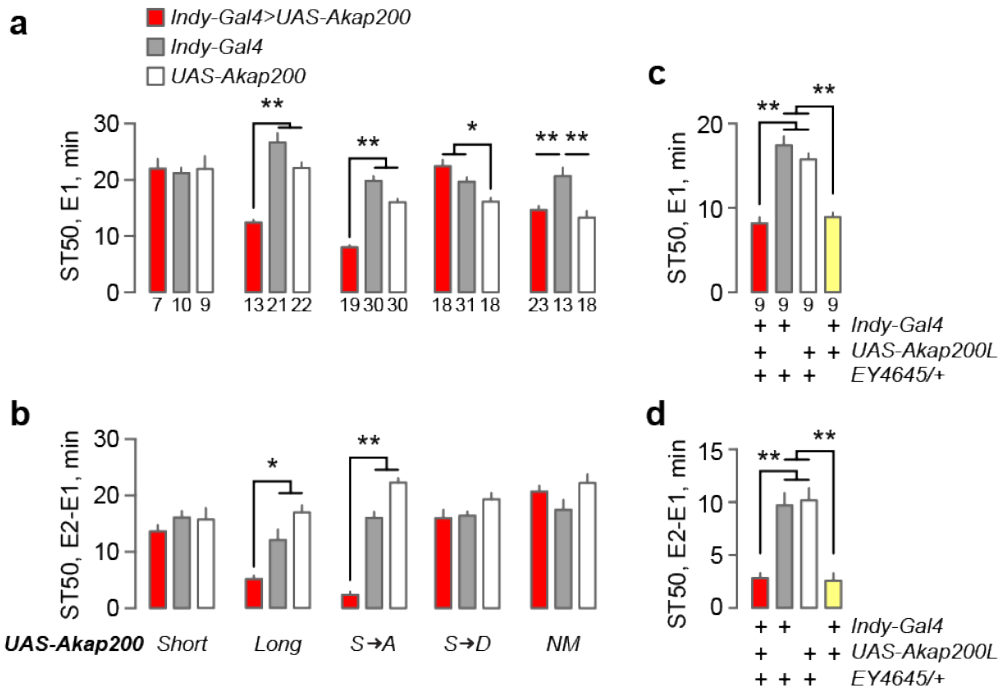


Figure 13. **Increased expression of Akap200 in perineurial glia increases ethanol sensitivity and decreases ethanol tolerance. a**, **b**. Ethanol sensitivity (**a**) and tolerance (**b**) when either wild-type or mutated Akap200 transgenes were expressed in perineurial glia of wild-type flies. Akap200 sequence changes were S->A: PKC non-phosphorylatable; S->D: PKC pseudo-phosphorylated; NM: myristoylation blocked. **c**, **d**. Ethanol sensitivity (**c**) and tolerance (**d**) in flies with reduced wild-type (*Akap200*^{EY4645}/+) and overexpressed Akap200L did not affect the overexpression phenotypes. One way ANOVA/Tukey's, the number of groups tested is indicated below each bar for all panels in figure. *$p < 0.05$, **$p < 0.01$.

4.6 PKA and Calcium Regulate Ethanol Tolerance in the Perineurial Glia

Akap200 physically interacts with the PKA RII regulatory subunit PKA-R2, the calcium binding protein calmodulin (CaM), and actin, and it is phosphorylated by PKC (Li et al., 1999; Rossi et al., 1999). We asked if PKA and intracellular calcium levels contribute to ethanol tolerance in the perineurial glia. RNAi-mediated reduction of *Pka-R2* in the perineurial glia decreased ethanol sedation sensitivity and decreased ethanol tolerance (**Figure 14a,b**). Overexpression of the PKA catalytic subunit (Pka-C), either the wild-type or a constitutively active form, had similar behavioral effects to loss of *Pka-R2*. Both manipulations of PKA are predicted to increase PKA activity. This suggests that Akap200 may promote ethanol tolerance by limiting PKA activity. Further, reducing CaM expression in the perineurial glia led to marked ethanol sedation resistance and a near absence of ethanol tolerance (**Figure 14a,b**), suggesting that a calcium-dependent process in the perineurial glia is important for ethanol responses. Consistent with this, reduced expression of the sarco/endoplasmic reticulum calcium-ATPase channel Cap60A or the inositol 1,4,5-trisphosphate receptor Itpr resulted in

decreased ethanol tolerance (**Figure 14a,b**). These results suggested that PKA and endoplasmic reticulum-mediated calcium signaling may be coordinated by Akap200 in the perineurial glia to promote ethanol sensitivity and tolerance.

Finally, we asked if altering the properties of the perineurial glia specifically during ethanol exposure affected behavior. We acutely depolarized the perineurial glia, increasing calcium influx, during ethanol exposure using the heat activated TrpA1 cation channel. TrpA1 activation did not alter ethanol sensitivity, but it strongly decreased ethanol tolerance (**Figure 14c,d**). These data demonstrate that the perineurial glia are actively involved in the development of ethanol tolerance.

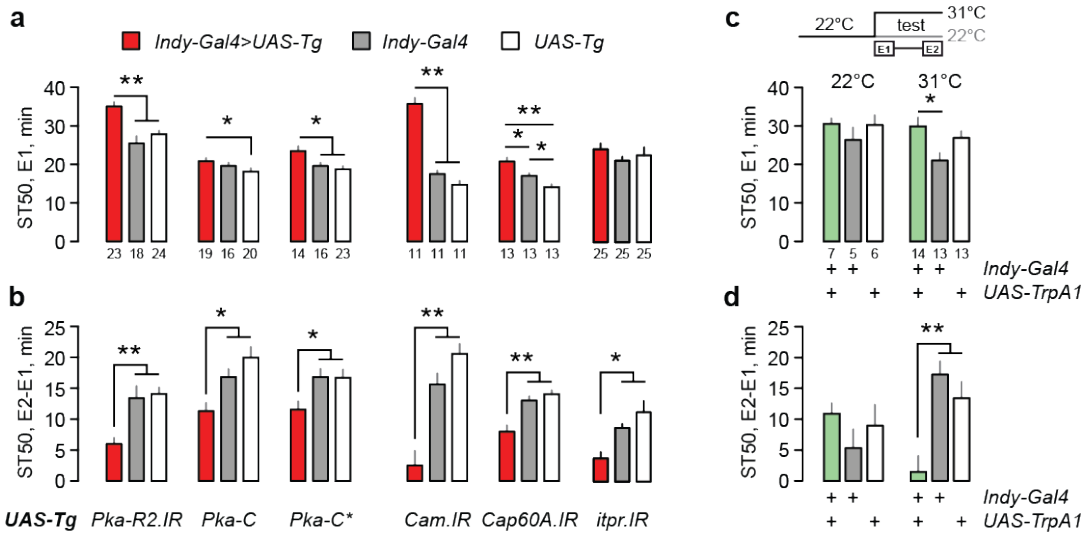


Figure 14. **Perineurial expression of proteins that interact with Akap200 is required for normal ethanol sensitivity and tolerance**. **a**,**b**. Ethanol sedation sensitivity (**a**) and tolerance (**b**) for flies expressing the indicated transgenes (*UAS-Tg*) in perineurial glia. *Pka-R2.IR*: PKA regulatory subunit RII RNAi; *Pka-C*: wild-type PKA catalytic subunit; *Pka-C**: constitutively active PKA catalytic subunit; *Cam.IR*: calmodulin RNAi; *Cap60A.IR*: SERCA RNAi; *itpr.IR*: InsP3R Itp-r83A RNAi. **c,d**. Ethanol sedation sensitivity (**c**) and tolerance (**d**) for flies expressing the TrpA1 cation channel in perineurial glia. Flies were raised at 22°C (TrpA1 off), and held at 22°C (control) or shifted to 31°C (TrpA1 on, experimental) just prior to commencement of ethanol vapor exposure. One-way ANOVA/Tukey's or Kruskal-Wallis/Dunn's. Number of groups tested is indicated below each graph. *$p < 0.05$, **$p < 0.01$.

4.7 Discussion

We show that the outer cellular layer of the Drosophila blood-brain barrier, the perineurial glia, is critical for the development of ethanol tolerance. Prior work shows conclusively that ethanol tolerance is due to changes in neuronal excitability and synaptic plasticity (Ghezzi and Atkinson, 2011; Lovinger and Roberto, 2013). We propose that ethanol generates tolerance in part by eliciting a signal that leads to A kinase anchoring protein-dependent changes in blood-brain barrier morphology and

function. Perineurial Akap200, membrane polarization, calcium regulation, and PKA activity all contribute to ethanol responses. These findings assign a function to the perineurial glia in the regulation of adult behavior. We suggest that a humoral-CNS communication pathway exists that is affected by ethanol and that transits through the barrier.

Maintenance of classical physical and chemical barrier functions is critical for normal behavioral responses to acute ethanol exposure. The fly G protein-coupled receptor Moody maintains septate junctions between subperineurial glia, forming the physical barrier. Moody mutants accumulate high molecular weight dyes in the CNS and show decreased ethanol sensitivity (Bainton et al., 2005). The fly Mdr65 transporter is also located in the subperineurial glia and is critical for selective exclusion of hemolymph molecules from the CNS (Mayer et al., 2009). Like Moody, loss of Mdr65 decreases ethanol sensitivity (unpublished observations). The perineurial glia are directly apposed to the subperineurial glia and both completely cover the adult CNS, suggesting that the barrier glia communicate to maintain barrier function. However, neither ethanol nor removal of Akap200 from the perineurial glia caused measurable changes in subperineurial glia-dependent barrier functions, indicating that Akap200 in the perineurial glia regulates ethanol behaviors by a distinct mechanism.

Acute ethanol exposure has pronounced effects on barrier-like properties and the cytoskeleton in culture-based models of the mammalian blood-brain barrier. Cultured brain endothelial cells form a confluent monolayer sealed with tight junctions. A 2 hr exposure to 50 mM ethanol causes changes in cell shape, increased paracellular permeability, and altered phosphorylation and distribution of tight junction-associated proteins (Haorah et al., 2005). Primary cultures of astrocytes treated with 100 mM ethanol for 10 min lose actin stress fibers and mislocalize the focal adhesion protein paxillin (Allansson et al., 2001; Guasch et al., 2003). Together these findings indicate that acute ethanol exposure can alter the cytoskeleton at the membrane, leading to decreased integrity of endothelial sheets and potentially decreased extracellular matrix adhesion of astrocytes. Our in vivo data is consistent with changes in cytoskeletal organization in the perineurial glia upon acute ethanol exposure, and Akap200 binds filamentous actin (Rossi et al., 1999). The delayed effects of ethanol on perineurial glia morphology in Akap200 mutants suggest that Akap200 may coordinate some of the biochemical pathways that ethanol engages to affect cytoskeletal organization. Akap200, like other AKAP proteins, may dynamically position protein complexes where they can respond to membrane-bound receptor activation in a localized fashion. Akap200 and the mammalian Akap12 (also known as SSeCKS and Gravin) lack sequence homology yet they physically interact with similar molecules, including PKA-RII, PKC, calmodulin, Src, and the actin cytoskeleton, and both proteins associate with membranes via N-terminal myristoylation and polybasic effector domains (Gelman, 2010). Akap12 expressed in astrocytes is induced by acute ethanol exposure, promotes blood-brain barrier formation in development, and tightens preformed endothelial barriers (Choi and Kim, 2008; Choi et al., 2007; Lee et al., 2003; Pignataro et al., 2013). Akap12 also regulates cell shape and motility in response to extracellular signals

by flattening membranes and decreasing chemotaxis, however the direct relationship between the extracellular signal and Akap12 is not known (Su et al., 2010; Weiser et al., 2008).

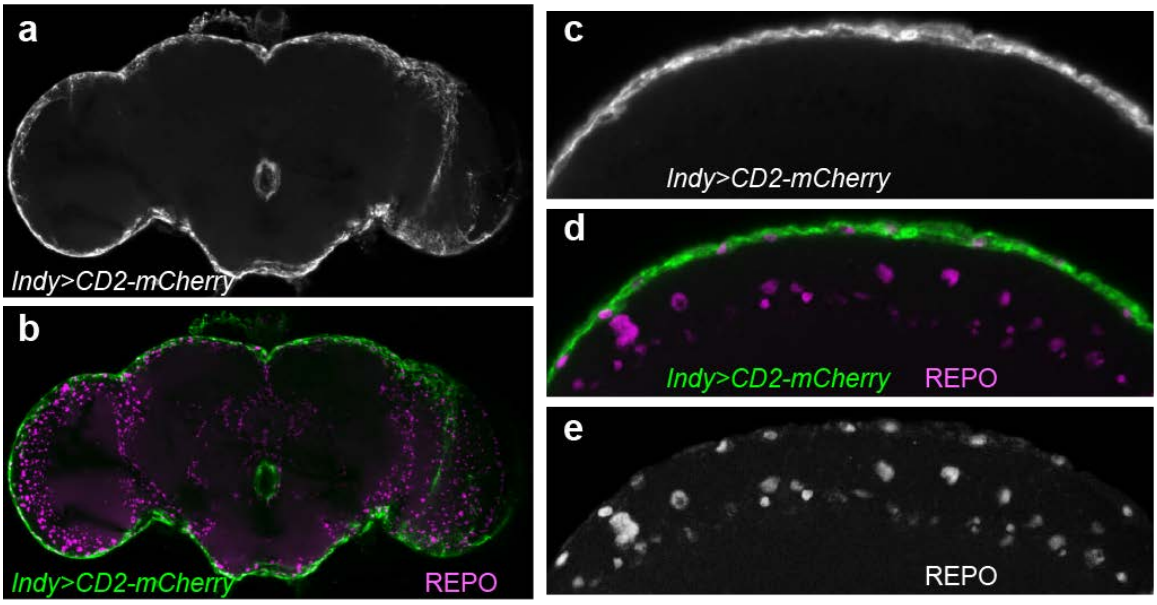
Acute ethanol exposure generally activates PKA signaling in mammals, and PKA regulates both the short and long term behavioral effects of ethanol (Ron and Messing, 2013). For example, deletion of PKA regulatory subunits or PKA pharmacological inhibition in specific mouse brain regions decreases sensitivity to the hypnotic effects of ethanol and increases ethanol consumption (Lai et al., 2007; Pandey et al., 2003; Thiele et al., 2000; Wand et al., 2001). In flies, PKA also regulates ethanol sensitivity: flies lacking or expressing a dominant negative PKA regulatory subunit show decreased ethanol sensitivity (Park et al., 2000; Rodan et al., 2002). Furthermore, genetic manipulation of adenylyl cyclase activity suggests that neuronal PKA signaling promotes the development of ethanol preference in Drosophila (Xu et al., 2012). Our results show that increasing PKA activity, like decreasing Akap200 expression, specifically in the perineurial glia decreases ethanol sensitivity and tolerance. These results suggest that Akap200 may promote ethanol tolerance by sequestering inactive PKA. Alternatively, Akap200 may limit the spatial distribution of activated PKA to achieve high fidelity signaling. Our results, combined with others, point to cell type specific roles for PKA signaling in the brain in ethanol behaviors (Rodan et al., 2002; Ron and Messing, 2013).

Cytoplasmic calcium levels in glia are mostly controlled by the endoplasmic reticulum. Calmodulin binds to Akap200 in the presence of calcium, displacing F-actin (Rossi et al., 1999). Calmodulin and endoplasmic reticulum stores of calcium in the perineurial glia are important for promoting ethanol sensitivity and tolerance, suggesting that calcium homeostasis may affect calmodulin and F-actin binding to Akap200. In mammals, elevation of intracellular calcium levels causes Akap12 to leave the membrane and become cytoplasmic or perinuclear (Schott and Grove, 2013). It is not yet known if ethanol exposure increases cytoplasmic calcium in the perineurial glia. However, experimentally increasing intracellular calcium during ethanol exposure led to decreased ethanol tolerance.

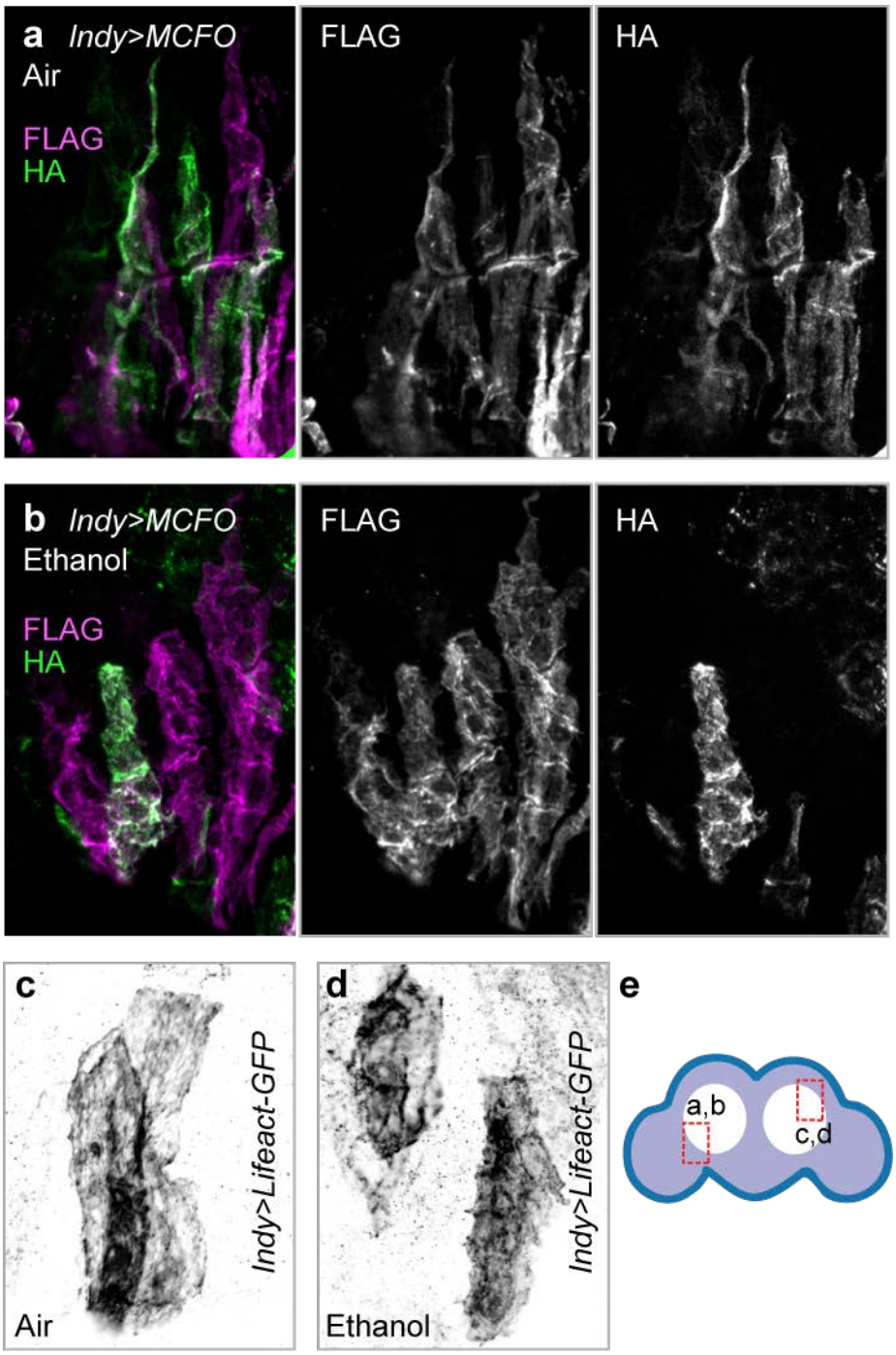
Several blood-brain barrier intercellular signaling pathways have been described for neural development and adult physiology in both mammals and insects (Alvarez et al., 2013). All the currently described signals in insects originate in the periphery to affect the CNS. First, nutrient availability in the hemolymph triggers production of the insulin-like molecule Dilp6 by the subperineurial glia to promote neuroblast proliferation in larvae (Spéder and Brand, 2014). It is notable that insulin regulates ethanol sensitivity in flies (Corl et al., 2005). Second, lipoprotein particles produced by the fat body adipose tissue cross the blood-brain barrier via glial-expressed LRP receptors, possibly to deliver signaling molecules to the CNS (Brankatschk et al., 2014). Endocannabinoids, which modulate ethanol behaviors in mammals, are carried on Drosophila lipoproteins (Khaliullina et al., 2015; Pava and Woodward, 2012). Third, hemolymph levels of octopamine, thought to function like mammalian epinephrine and

norepinephrine, are increased by stress, and octopamine receptors are likely present on the blood-brain barrier (Davenport and Evans, 1984; Schofield and Treherne, 1985). Octopamine is required for the development of ethanol tolerance (Scholz et al., 2005). Finally, the subperineurial glia produce sex-specific signals to regulate male courtship (Hoxha et al., 2013). These findings indicate the diversity of roles played by the blood-brain barrier beyond classical partitioning of the humoral and neural compartments, and suggest possible signaling mechanisms used by ethanol to regulate behavioral plasticity.

4.8 Supplemental Figures



Supplementary Figure 1. ***Indy-Gal4*** **brain expression is limited to the perineurial glia. a**, **b**. Whole mount *Indy-Gal4/+;UAS-CD2mCherry/+* (green) brain counterstained with antibodies to the glial nuclear protein REPO (magenta), 20 μm compressed stack. **c**, **d**, **e**. 2 μm section of lateral optic lobe at higher magnification shows no REPO-positive cells external to the *Indy-Gal4*-expressing layer.



Supplementary Figure 2. **Ethanol effects on individually labeled perineurial cells**. **a**,**b**. Stochastic labeling of individual perineurial cells using Muliticolor FlpOut in flies either sham treated with humidified air (**a**) or a sedating dose of ethanol vapor (**b**), and a subsequent 4 hr recovery. Independent recombination events were detected with either HA or FLAG tags. **c**,**d**. Representative discretely labeled perineurial cells expressing the actin binding Lifeact-GFP protein, in flies sham treated with humidified air (**c**) or with ethanol vapor (**d**). Confocal sections are inverted to highlight the actin pattern. **e**. Locations for the perineurial glia shown in this figure.

CHAPTER 5: CONCLUSIONS

5.1 Short-term Goals

The short-term goal of this work was to identify a role for glia in ethanol behaviors. While this was unsuccessful for the astrocytes at moment, follow up work should be done to investigate these cells, especially once Gal4 drivers become available that label specific neuropil-associated astrocytes. Furthermore, the results of the Akap200 work in the perineurial glia have opened new avenues of research into how these cells are interacting with neurons for the behavioral phenotypes we have observed, but also with the other glia cells that are physically between the neurons and the perineurial glia. Current work has started to suggest a signaling cascade involving actin and calcium but more research should be performed to better understand these interesting cells.

5.2 Long-term Goals

Future studies in the glia-ethanol field should include potential drug therapies for AUDs. As there are no current drug therapies in place to combat AUDs research into glia as a drug target is imperative. Understanding the relationship glia have with alcohol is crucial to this outcome and the work presented in this thesis is barely a starting point into the understanding of these cells. With the increasing genetic knowledge of these cells, new tools will be developed to better manipulate these cells and allow for understanding of their inner workings, and the disappearance of the “black box” figures that are all too common in the literature.

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