2022

Application of Confocal Microscopy To Study the Neural Mechanisms Underlying Insect and Rodent Behavior

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APPLICATION OF CONFOCAL MICROSCOPY TO STUDY THE NEURAL MECHANISMS UNDERLYING INSECT AND RODENT BEHAVIOR

A dissertation submitted to
The Graduate College of
Marshall University
In partial fulfillment of
The requirements for the degree of
Doctor of Philosophy
In
Biomedical Research
By
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Approved by
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Marshall University
May 2022

APPROVAL OF DISSERTATION

We, the faculty supervising the work of Christian Michelle Harris, affirm that the dissertation, *Application of Confocal Microscopy to Study the Neural Mechanisms Underlying Insect and Rodent Behavior*, meets the high academic standards for original scholarship and creative work established by the Biomedical Research Program and the Graduate College of Marshall University. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.

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DEDICATION

I would like to dedicate this dissertation to my parents Mr. and Dr. Torye and Lisa Harris as well as my grandparents, Judge and Mrs. Roy and Lucille King, and Mr. and Mrs. Melvin and Yvonne Harris. Throughout my life my parents and grandparents were there with me, giving me guidance, encouragement and always pushing me to be my best. During my matriculation through Marshall, I lost three of my four grandparents, who were my best friends. In their final days each of them told me to make sure I finish. Well here I am, I hope I've made you guys proud. I love you forever and thank you for all that you all have done for me.

ACKNOWLEDGEMENTS

First, I want to thank my Lord and savior Jesus Christ, as without him I am nothing. I would like to thank my parents Mr. and Dr. Torye and Lisa Harris, thank you for investing in me throughout my life, thank you for never allowing me to take the easy road, thank you for continually supporting and loving me. I love you both so much and I would not be where I am and who I am without you all, thank you for being the best parents in the world. I would like to thank my grandparents Melvin D. and Yvonne Harris, and Roy W. and Lucille A. King, for supporting me and loving me you all played instrumental roles in my life, and I hope I have made you all proud. I would like to thank my brother Brandon and my sister-in law Tiffany for their constant thoughts, prayers, and support. I would like to thank my dog Boomer and my dog niece Sophie for always bringing a smile to my face and being a form of solace. I would like to thank Dr. Andrey Semichaevsky who introduced me to research and was instrumental in helping me get my first research internship with Dr. Stephanie Law at the University of Delaware. I would like to thank my friends, extended family, and loved ones particularly, Terrill Barringer, Skylar Cooper, Cecilia and Tanner Bahkshi, Arrin and Kevin Brooks, Khadija and Aisha Muhammad, Justice Lynch, LaDajha Langley-Hart, Arianna Polk, Nichelle Norman, Gianna Gordan, Lisa Allen, Marie Murphy, Marie Redd, Cynthia Jones, friends from Lincoln University, Pastor John Porter and my first Baptist church family, Bishop James R. Wright and my New Progressive Cathedral Church of God in Christ family, The Eureka #12 Chapter of the Order of the Eastern Star, The Huntington Chapter of the Links, Incorporated, and The Delta Nu Omega Chapter of Alpha Kappa Alpha Sorority, Incorporated, for their constant love and support. I would like to thank my committee, Louise Risher, Ph.D., Sasha Zill, Ph.D., Eric Blough, Ph.D., Michael

Norton, Ph.D., and Brandon Henderson, Ph.D. for their advice, dedication, and guidance during my matriculation.

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ABSTRACT

Posture and walking require support of the body weight, which is thought to be detected by sensory receptors in the legs. Specificity in sensory encoding occurs through the morphological properties of the sense organs (numerical distribution, receptor size) and their physiological response characteristics. These studies focus upon campaniform sensilla, receptors that detect forces as strains in the insect exoskeleton. To study the morphology of campaniform sensilla, the sites of mechanotransduction (cuticular caps) were imaged by light and confocal microscopy in four species (stick insects, cockroaches, blow flies and Drosophila). These data indicate that the gradient (range) of cap sizes may most closely correlate with the body weight. These studies support the idea that morphological properties of force-detecting sensory receptors in the legs may be tuned to reflect the body weight. Overall, this study indicates that the morphological properties of the sense organs are specifically tuned to provide information needed for postural stability and successful locomotion.

Key Words: Force, Insect, Campaniform, Response, Sensitivity, Gradient

CHAPTER 1

INTRODUCTION

Signals from sense organs that encode forces in the legs have been shown to be integral to nervous system control of walking in both vertebrates (including humans) and invertebrates (Duysens et al., 2000; Tuthill & Wilson, 2016). In vertebrates, forces that resist muscle contractions are encoded at the musculo-tendinous junction by Golgi tendon organs (Prochazka & Gorassini, 1998). In insects, forces are detected by campaniform sensilla, which encode the effects of muscle contractions and loads as strains in the exoskeleton (Tuthill & Wilson, 2016; Zill et al., 2000). The effects of these diverse sense organs are remarkably similar in both vertebrate and invertebrate systems: the nervous system uses force information to adjust the magnitude and timing of contractions of leg muscles and to determine phase transitions (stance to swing) during walking. These similarities are likely a consequence of the common biomechanics underlying leg use.

While there is extensive literature on the response properties of force receptors, there are several questions that remain unanswered; 1- Tuning of force receptors to the range of forces- The range of forces acting upon the legs varies widely, both in individual development and among different species. It is not known if the responses of force detecting sense organs are tuned to the range of forces generated by the animal (Chapter 2). 2- Homologies in different species - Previous studies have characterized the campaniform sensilla located on the legs in a number of species of insects. However, it is not known if there are homologous groups that can fulfill the same basic functions in different species. Our current work has found unique differences in the size and number of these force receptors that correlates with the size of the

insect. We believe that size and number of force receptors may be correlated response and compensatory ability of the insect sensory system.

Background

Insects have been widely studied due to the numerical simplification (i.e. less complex components) of their nervous system and, recently, the availability of genetic tools to explore mechanisms underlying behavior. In insects, forces in the limbs are detected by Campaniform sensilla as strains in their exoskeleton. Each sensillum consists of a sensory neuron whose dendrite inserts into a cuticular cap at the surface of the exoskeleton. In most receptors, the cap is asymmetrical and previous studies have shown that the sensilla respond best to compressions that occur perpendicular to the cap long axis or tension parallel to the axis (Ridgel et al., 2000). The cap serves as the site of mechanotransduction, which is now thought to be mediated by NOMPc ion channels on the sensory dendrite (Sun et al., 2019). Campaniform sensilla can occur in isolation but most often organize in groups with definable cap orientations. Individual receptors can encode force increases or decreases according to the orientation of their cuticular cap and the vectoral direction of strains in the exoskeleton. Previous studies have also shown that the range of force detection in individual sensilla is correlated with the size of the extracellularly recorded action potential and that receptors with smaller cuticular caps have small potentials (Zill et al., 2013; Zill et al., 1999). Activities of campaniform sensilla have also been recorded in freely walking insects (Noah et al., 2001; Zill et al., 2009). Mechanical forces on the body are consequences of self-movement, which elicits different sensory response discharges based on specific movement (Tuthill & Wilson, 2016). Discharges of campaniform sensilla have been incorporated into simulations and robotic controllers for insect walking although in some models and simulations of walking, the dynamic component has only been considered to function in the

period after leg contact, not in the detection of forces throughout the stance phase (Daun-Gruhn & Büschges, 2011; Dürr et al., 2019), and are used by Szczecinski et al. (2017b; 2017a) in their robotic insect models.

Force Sensing In Posture And Locomotion

Monitoring forces via sense organs play an essential role in the control of posture and locomotion (Duysens et al., 2000). There are receptors in the leg that detect forces or signal muscle length and rate of change in length such as muscle stretch receptors (muscle spindles), tendon organs (Golgi tendon organs), joint receptors, and cutaneous mechanoreceptors. However, the focus of this work is on sense organs that encode forces in the leg that provide the system with data about the direction, rate, and magnitude of load. This information can be used to generate responses to postural perturbations and in adapting in walking movements (Ridgel et al., 2001). Perturbations occur naturally while maintaining posture, the body adapts with compensatory responses in order to maintain stability. In walking, receptors that monitor forces have two major functions: 1- provide information to adjust the levels of motorneuron activities to load during stance; 2- act as signals to determine the timing of transitions between stance and swing phases (Duysens et al., 2000). In a number of animals, transition between swing and stance is strongly correlated with changes in load which mediate the feedback systems and plays a crucial role in timing of leg lift and touch down. These feedback systems utilize information from proprioceptors (ex. Golgi Tendon Organs) encoding load that permit swing when the leg is unloaded; however, specific signal detail or their motor effects are not fully known. In insects studies have shown in inactive stick insects, receptors produce negative feedback. In “active” stick insects, sense organs elicit positive feedback reflexes (Tuthill & Wilson, 2016; Zill et al., 2000). Force receptor activity in stance phase may potentially prevent initiation of swing phase.

Regulation of forces that counter body loading has also been demonstrated in control of posture and walking of invertebrates (Zill et al., 1993). Forces applied to the body can have effects on body position and elicit responses to counter the load stemming from joint angle receptors (hair plates) (Cruse et al., 1993). Force receptors in invertebrates can adjust motor outputs to load during walking. Sudden decreases in force can affect timing of phase transitions eliciting early swing movements (Cruse, 1990). In summary, these studies support the idea that force receptors play a vital role in regulating posture and walking.

Force sensing in insects

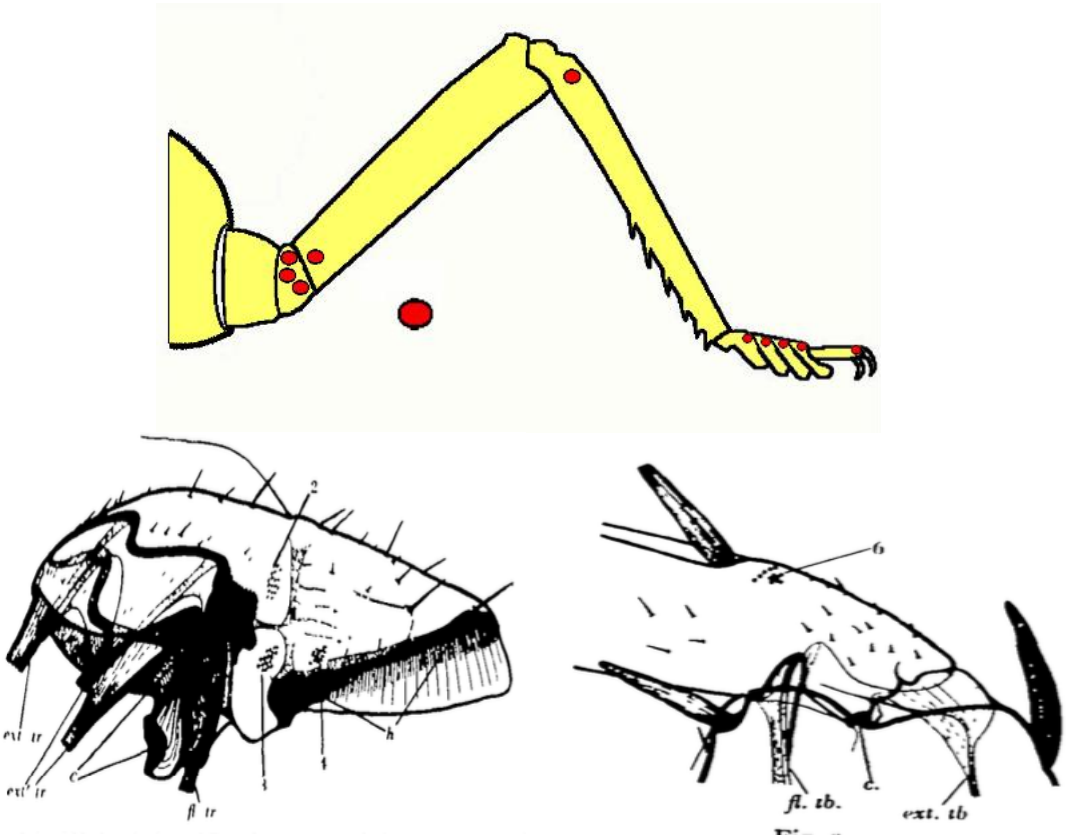

Figure 1: Tibial and trochanteral campaniform sensilla groups

Figure A- a schematic of where both tibial and trochanteral groups of campaniform sensilla. B- A drawing of trochanteral campaniform sensilla from JWS Pringle (1938). C- A drawing of tibial campaniform sensilla from Pringle (1938).

As previously mentioned, insects have sense organs called campaniform sensilla that contains a sensory neuron whose dendrite inserts into a cap at the surface of the cuticle which monitor forces via strains that occur in the exoskeleton (Pringle, 1938). The responses of individual receptors are correlated with the orientation of the cuticular cap which is found at the surface of the exoskeleton. In cockroaches, the tibial groups consist of proximal and distal sensilla which have two perpendicular orientations (Figure 1A/1C). When bending occurs discharges encode the direction, magnitude and rate of change of force (Ridgel et al., 1999; Zill & Moran, 1981a, 1981c). Tibial sensilla also show phasic discharges when force decreases. When forces are applied to the tibia that mimic ground reaction forces or the force exerted on the body from the ground that it came in contact with, the proximal sensilla respond to force increases while distal sensilla respond to force decreases (Ridgel et al., 1999). Leg muscle contractions produce specific firing in campaniform sensilla: resisted contractions of the tibial extensor excite the distal and inhibit the proximal sensilla (Zill & Moran, 1981a, 1981c; Zill et al., 1981). In freely standing and walking animals, strains generated by contractions of the trochanteral extensor muscle on the tibia sensilla depend upon the joint angle (Figure 1A/1B) (Noah et al., 2001). The proximal and distal sensilla have varying functions during walking (Ridgel et al., 1999). The proximal sensilla fire when the insect is in upright position and burst in stance phase of walking (Zill & Moran, 1981c; Zill et al., 1981). When the leg first contacts the surface, the proximal sensilla begin to fire yet will not reach maximum firing rate until in full

stance then continually decrease until the end of stance (Zill & Moran, 1981c). Proximal receptor firing follows the onset of bursting of the trochanteral extensor slow depressor motor neurons (Ds) while the slow tibial extensor motor neurons (SETi) becomes active later in stance (Noah et al., 2001). Subsequent proximal sensilla activity is reciprocally related to the frequency of discharge of SETi. Leg extensor muscles potentially have different effects upon the proximal discharge. Proximal firing directly follows activity of the trochanteral extensor; however, their activity can be inhibited by the tibial extensor (Zill & Moran, 1981c; Zill et al., 1981). Distal sensilla firing occurs as a short burst toward the end of stance prior to the onset of swing (Watson & Ritzmann, 1997). Strains generated by the tibial extensor show that it is active late in stance and provides propulsive force. Previous research shows that discharges of distal receptors at the end of stance phase just prior onset of leg lift, are a result of decreasing forces on the leg (Zill & Moran, 1981c). Previous kinematic data show flexor firing occurs in a specific pattern, small units initiate firing just prior to leg lift, while large units are recruited at onset of or during swing (Noah, 2003). Sensory feedback that occurs towards the end of stance can contribute to the recruitment patterns and acceleration of firing in the flexor muscle. Thus, signals of campaniform sensilla during the stance phase may both adjust motor outputs to load and contribute to the onset of leg lifting in swing.

Structural/Functional Correlates Of Campaniform Sensilla

A variety of studies have examined the relationship between the morphology and location of campaniform sensilla and their functions in behavior. J. W. S Pringle was one of the first to identify and develop nomenclature for the groups of receptors found on the legs of the American cockroach (*Periplaneta Americana)* (Pringle, 1938). The individual sensilla are typically found on the tarsi, while receptors are arranged in groups on the trochanter and femur. Pringle

identified 53 sensilla in groups 1, 2, 3, 4 and 5 located on the trochanter and femur while group 6 is located on the tibia, and groups 7-11 on the tarsus (Pringle, 1938). Werner Gnatzy further identified groups of sensilla in blowflies (*Calliphora vicina)*. He identified 52 groups and 24 different types of sensilla in the leg, antenna, forewing, and haltere (Gnatzy et al., 1987). Calliphora have groups similar to the cockroach; however, Calliphora and Drosophila are missing group 2. Recently, Gesa Dinges using confocal and scanning electron microscopy (SEM) investigated the morphological distribution of campaniform sensilla on the legs based on the identification of groups from Gnatzy (Dinges, 2018). Using computed tomography (CT) allowed for precise determinations of cap orientations and relative locations which are typically difficult to represent with solely SEM. Her study focused on identifying the locations and orientations of the various groups in the prothoracic, mesothoracic and metathoracic legs and noted that there were only small variations in number of sensilla within a group in Diptera.

The potential functions of campaniform sensilla in behavior and structures of the leg segments are discussed by Pringle (1938), Merritt & Murphey (1992), and Frantsevich & Gladun (2002). Pringle (1938) and Frantsevich & Gladun (2002) described the functions of the trochanter, coxa, and femur. The coxa is a part of the attachment point or the "hinge" of the leg to the body, it is articulated at an extrinsic joint with sclerotized condyles and depending on species the shape can vary, (Frantsevich & Wang, 2009; Pringle, 1938). The trochanter is a smaller segment between the coxa and femur, it has two condyles which limit the movement to one plane. It also has a reductor femoris muscle which inserts into the femur (Frantsevich & Wang, 2009; Pringle, 1938). The femur is attached to the trochanter by an intrinsic hinge and forces generated by the intrinsic pulling on the reductor muscle are balanced by the elastic restoring force (Pringle, 1938). Frantsevich & Gladun (2002) further characterized the

morphology of the coxa, their sensilla and body size. He studied over 200 species comparing their leg positions during flight. He then imaged their coxo-trochanteral joints (CT- joint) and counted the number of hair and campaniform sensilla. Frantsevich & Gladun (2002) then compared the number of sensilla in various species and found that there is no simple correlation between the number of sensilla and the size of the insect. D.J. Merritt and R.K. Murphey (1992) described the sensory receptors that innervates each sensillum. Using fluorescent dye staining they imaged the thoracic ganglion, which can be partitioned into two layers: layers of neuropile, which are on the ventral surface of the neuromere, the second layer called the intermediate makes up a majority of the central portion of the ganglionic neuropile (Merritt & Murphey, 1992). The leg sensilla project into the intermediate layer of the neuropile. They were able to identify the specific segregation of axonal arborizations in the CNS although there was no clear correlation between the size of the projections and the mass of the animal. The relationship between the properties of force receptors and the weight of the animals remains largely unresolved. In the study described in this dissertation, we examined and compare the morphology of groups of campaniform sensilla in a number of insect species. The arrangement of campaniform sensilla suggest that there are homologous groups found on the trochanter and femur in cockroaches, stick insect and flies. Some groups have similar structure and location in all species, such as groups 3 and 4 while other groups such as group 5 are specialized. Furthermore, measurements of the size of the cuticular caps showed that the mass of the animal was related to the gradient of sizes of the cuticular caps of the receptors within individual groups. In addition, study of the receptor caps in first instar (newly molted from eggs) cockroaches suggested that the gradient of cap sizes also expands in the development of individual animals as they increase in size and mass in development. Although not well studied, these relationships

between the sizes of receptors and the mass and forces generated by an animal may also apply to vertebrates, as Golgi tendon organs occur in diverse sizes and numbers. Further research is needed to establish a clear relationship between the structure and response properties of force receptors and the magnitude of forces generated by all animals of diverse sizes and weights.

Conclusion

Ultimately, our work will provide new and valuable data about how force information is encoded, and also gives insight into how these signals are incorporated into walking behavior. Receptors, such as Golgi tendon organs and campaniform sensilla, are considered to be proprioceptors that simply encode force but previous studies suggest that their responses properties are specifically adapted to the control of forces encountered and generated by the animal. The potential adaptation of the range of receptor caps sizes to the weight of the animal has not previously been determined and will provide data about how the mechanisms of sensory transduction are tuned to the range of forces in individual development and among different species. Possible variation in receptor morphology in front, middle and hind legs will also be studied in different species. The results of these experiments have potential applications in the areas of neurobiology, prosthetics and bio-robotics. Understanding the role of force is important in prosthetics, as the device applies force to the body which changes the way it interacts with the body. The older artificial legs were largely passive devices but modern prostheses that are being developed are adaptable and controlled by microprocessors. However, many prosthetics only utilize signals as “switches” to indicate ground contact without incorporating information about force magnitude or utilizing signals of force dynamics, which recent studies have shown can potentially lead to greater adaptability in use of artificial limbs. Our results on force sensing have

also been incorporated into models of insect walking and applied to legged robots (Szczecinski et al., 2017b; Szczecinski et al., 2017a).

CHAPTER 2

GRADIENTS IN MECHANOTRANSDUCTION OF FORCE AND BODY WEIGHT IN INSECTS

A manuscript published in *Arthropod Structure and Development*

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Abstract

Posture and walking require support of the body weight, which is thought to be detected by sensory receptors in the legs. Specificity in sensory encoding occurs through the numerical distribution, size and response range of sense organs. We have studied campaniform sensilla, receptors that detect forces as strains in the insect exoskeleton. The sites of mechanotransduction (cuticular caps) were imaged by light and confocal microscopy in four species (stick insects, cockroaches, blow flies and Drosophila). The numbers of receptors and cap diameters were determined in projection images. Similar groups of receptors are present in the legs of each species (flies lack Group 2 on the anterior trochanter). The number of receptors is generally related to the body weight but similar numbers are found in blow flies and Drosophila, despite a 30-fold difference in their weight. Imaging data indicate that the gradient (range) of cap sizes may more closely correlate with the body weight: the range of cap sizes is larger in blow flies than in Drosophila but similar to that found in juvenile cockroaches. These studies support the idea that morphological properties of force-detecting sensory receptors in the legs may be tuned to reflect the body weight.

Introduction

Detection of the magnitude and dynamics of forces is integral to motor control in both vertebrates (Lin et al., 2019) and invertebrates (Zill et al., 2018). Posture and walking require countering and supporting the weight of the body (Ivanenko & Gurfinkel, 2018) and a major source of forces acting on the legs is the body mass (Chiel et al., 2009). Control of the position and movement of the center of mass (via center of pressure) is a central component in many models and simulations of control of posture and walking in animals and robots (Szczecinski et al., 2014; Szczecinski et al., 2017a; Ting et al., 2015). The effects of body weight are thought to

be detected by sensory systems that encode forces in the legs. However, it is not clear how sensory systems are tuned to detect changes in the effects of body mass or whether the information is processed to produce an integrated signal of body weight. Previous studies suggest that control is distributed and that body weight may not be calculated by discrete processes but, instead, is an emergent property of the system (ex.(Horak et al., 1997)). Specificity of information about body weight could occur by two mechanisms 1) the distribution of receptors detecting forces and 2) the range of sensitivities of sensory neurons. For example, in vertebrates, Golgi tendon organs, which detect forces of resisted muscle contractions, are known to be most numerous in large antigravity muscles, while much smaller numbers of receptors are found in muscles that generate fine movements (Jami, 1992). Although less well studied, Golgi tendon organs also vary in size (Goldfinger & Fukami, 1982). Both the length and diameter of tendon organs are correlated with the number of muscle fibers that attach to the organ capsule (and implicitly the magnitude of forces). This could form a morphological basis for range fractionation, which is present in responses of tendon organs (Fukami & Wilkinson, 1977), although the correlation between receptor size and response range has not been examined.

In insects, forces in the limbs are detected as strains in the exoskeleton by mechanoreceptive campaniform sensilla (Zill et al., 2004). Each sensillum consists of a sensory neuron whose dendrite inserts to a cap at the surface of the cuticle. Mechanotransduction is thought to be mediated by NOMPc ion channels concentrated near the tip of the sensory dendrite (Sun et al., 2019). The cap is linked to the surrounding cuticle by a membrane and collar (Moran et al., 1971). Previous studies have shown that the membrane surrounding the cap is compliant (Chapman et al., 1973; Chapman et al., 1979) and shows autofluorescence in UV illumination (Zill et al., 2000), while the cap itself is composed of exocuticle and does not fluoresce. In most

sensilla, the cap and underlying cuticular collar are asymmetrical (Moran et al., 1971; Moran & Rowley, 1975). Asymmetry is associated with directional sensitivity, as receptors respond best to forces that produce compressions perpendicular to the cap long axis or tensions parallel to the axis (Ridgel et al., 2000). The range of forces detected by individual sensilla is generally correlated with the size of the extracellularly recorded action potentials and receptors with smaller cuticular caps have small potentials (Zill et al., 2013; Zill et al., 1999).

Campaniform sensilla frequently occur in isolation but are most often organized in groups with definable cap orientations (Pringle, 1938). Groups of sensilla were first identified (based upon their location) on leg segments of the cockroach and given sequential numbers by Pringle (1938) (Fig 1C). Sensilla on the legs of the stick insect (*Carausius morosus*) were characterized by Hofmann and Bässler (1982, 1986) who used a different system of nomenclature. Campaniform sensilla on the legs of blow flies were extensively studied and identified by Werner Gnatzy and colleagues (Gnatzy et al., 1987) while the receptors in Drosophila were characterized and groups identified by Merritt and Murphey (1992). Each of those studies used different systems of nomenclature. Previous studies also directly demonstrated that some groups of sensilla on the legs can encode the effects of changes in body weight, produced via small magnets placed over the center of mass (Keller et al., 2007; Noah et al., 2004; Quimby et al., 2006).

Study of the development of campaniform sensilla in cockroaches, which are hemimetabolous and increase in size and mass in successive molts, showed that there is an increment in the number and mean size of cuticular caps (Ridgel et al., 2003). This finding was consistent with the idea that the number and morphological properties of receptors are tuned to the increase in forces encountered by the animal in development. However, a study of the

cuticular caps in flies (Frantsevich & Gladun, 2002) showed that the number of cuticular caps was independent of the size of the animal in many species (Cyclorrhapha, ~72,000 species, including blow flies and Drosophila). The present study was, therefore, undertaken to: compare the morphology of groups of campaniform sensilla in different insect species (cockroaches, stick insects, blow flies and Drosophila) of widely varying size and mass. The size of cuticular caps of campaniform sensilla was also examined in juvenile cockroaches and compared to flies to determine if there were consistent morphological parameters that reflected tuning to body weight.

Methods

Studies were performed on: adult stick insects (female *Carausius morosus*), raised in animal colonies at the University of Cologne); cockroaches (*Periplaneta americana)* and fruit flies (*Drosophila melanogaster*), obtained from Carolina Biological Supply; and blow flies (*Calliphora vomitoria*), purchased as larvae from a commercial supplier (USMantis). Juvenile cockroaches were kindly provided by Dr. Peter Kloppenburg (University of Cologne) or purchased from Carolina Biological Supply.

Studies were performed on hind legs of flies and cockroaches, and on middle and hind legs of stick insects, as previous physiological studies had been performed on those legs. Data are plotted for stick insect middle legs but comparison of sensilla in scanning electron micrographs (kindly provided by Annelie Exter and Josef Schmitz, Bielefeld University) showed equivalent numbers (data below) and cap sizes in both middle and hind legs.

To study the morphology of the cuticular caps, animals were first anesthetized with carbon dioxide or cold. Legs were removed and cut into segments, then immersed in 4% paraformaldehyde buffered fixative. Specimens from cockroaches and stick insects were first

placed in fixative for 0.5-1 hour to stabilize the exocuticle then immersed in 1 N sodium hydroxide for 4-24 hours to remove soft tissues. All specimens were then returned to 4% formaldehyde to complete fixation for a minimum of 24 hours. Leg segments containing campaniform sensilla were excised and placed in a clearing agent (Conray). Specimens were mounted on slides that permitted viewing from both surfaces (drilled aluminum with coverslips on two sides). The cuticle containing campaniform sensilla was viewed in wholemount by light microscopy (Olympus BX60) and digitally imaged (OMAX A35180U3). Preparations were also studied by confocal microscopy (Leica TCS SP5 II microscope at the Marshall University Microscopy facility) through imaging of endogenous fluorescence of the cuticle (excitation 568 nm, emission 585 nm) (Zill et al., 2000). The number of caps was determined and cap long axis diameters measured in projection images using ImageJ software (NIH). Measurement points were set on the cuticular collar immediately surrounding the cap (Moran & Rowley, 1975). Identification of groups of sensilla in Drosophila was aided by diffusion of diI into fixed leg nerves and tissues (methods of (Zill et al., 1993)).

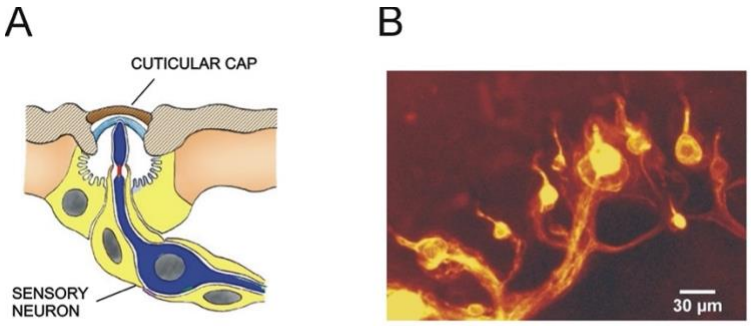
Determining the total number of sensilla required views that included all caps. In addition, the measurement of the cap size necessitated obtaining images taken normal to surface. This was facilitated by excising the cuticle, which tended to flatten the inherent curvature of the leg. The effect of flattening was minimal, as indicated by measurements taken from scanning electron micrographs of the sensilla of intact leg segments (images of stick insects kindly - provided by Annelie Exter and Josef Schmitz, Bielefeld University). These requirements limited the number of specimens that were included in the data analysis to images in which all sensilla could be visualized and their sizes accurately measured. The final data set was derived from 56 animals (12 stick insects, 12 adult cockroaches, 14 juvenile cockroaches, 9 blow flies, 9

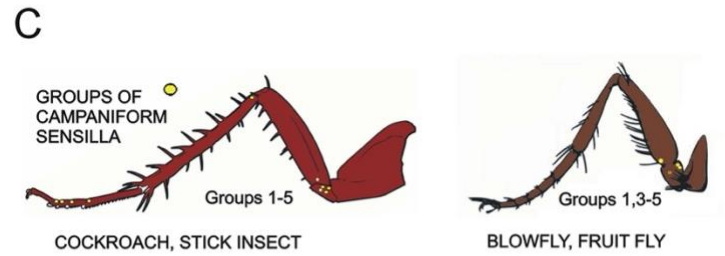
Drosophila). The orientations of sensilla relative to the long axis of the leg were estimated using cuticular landmarks. Due to their proximity, Groups 3 and 4 were measured together in the same preparations to compare the orientations of their cuticular caps. To obtain estimates of weight, insects were placed and weighed on a laboratory scale either as individuals or in small groups (Drosophila). All data were assembled on spread sheets (Excel) and plotted in software (Sigmaplot). Statistics were calculated using SPSS software (IBM).

Results

Identification of groups of campaniform sensilla in different species

Campaniform sensilla can be identified externally by their cuticular caps (Fig 2A). Sensilla are arranged in groups (Fig 2B) and the greatest concentrations occur in proximal leg segments (the trochanter and proximal femur, Fig 2C). The present study has found clear similarities in these groups in different insect species based upon their segmental location. Figure 2D is a table of groups arranged according to Pringle's numerical system for cockroaches (Pringle, 1938) and indicating similar groups in stick insects and flies (nomenclature of Hofmann and Bässler (1982); Gnatzy et al. (1987), Merritt and Murphey, (1992)). Groups 3 and 4 are located on the dorsal side of the trochanter and similar groups can be identified in all species examined in the present study. In addition, Group 2 is apparently present in cockroaches and stick insects but absent in blow flies and fruit flies.





D HOMOLOGOUS GROUPS OF RECEPTORS

Leg Segment	Cockroach (Adult, Juvenile)	Stick Insect	Calliphora (Blow Fly)	Drosophila (Fruit Fly)
Trochanter	Group 1	pCS	PG1	CS3
	Group 2	aCS	-	
	Group 3	dCS	AG1	CS8
	Group 4	dCS	AG2	CS5
Femur	Group 5	fCS	PG2-4	CS11

Figure 2: Identification of groups of Campaniform Sensilla in different species

A. Campaniform sensilla monitor forces as strains in the exoskeleton via the insertion of the dendrite of a sensory neuron to a cuticular cap. B. Many campaniform sensilla are organized into groups this image shows a diI fill of trochanteral Group 2 in a cockroach. C. Similar groups can be identified by location in cockroaches, stick insects and flies (blow flies, Drosophila). D. Nomenclature of groups - Groups of sensilla were first identified and numbered in the cockroach by Pringle (1938). Groups in similar locations can be found in flies, except for Group 2 on the anterior trochanter which is apparently absent in flies.

Structure of trochanteral Groups 3 and 4

The groups of campaniform sensilla on the dorsal surface of the trochanter (Groups 3 and 4) were similar both in location and in the arrangement of the cuticular caps. Figure 3A shows

confocal projection images and outline drawings of cuticular caps of trochanteral Groups 3 and 4 in stick insects, blow flies and Drosophila. The largest caps of Group 3 are oriented approximately perpendicular to the leg long axis, while the cap axis of large Group 4 receptors is approximately parallel to the axis. The mean difference in angles of Groups 3 and 4 was stick insects 82.8 +/- 5.9 SD degrees, blow flies 85.0 +/- 8.0 SD degrees and Drosophila 92.2 +/- 11.0 SD degrees. Asymmetry of the caps of smaller sensilla was less apparent in confocal projection images but sequential optical sectioning often demonstrated an oval shape in the cuticular collars. This constancy is of considerable interest as previous studies in cockroaches and stick insects have shown that the location and orientation of cuticular caps of these groups produces maximal sensitivity to forces in the plane of the coxo-trochanteral joint.

The number of sensillum caps in Groups 3 and 4 was higher in stick insects (Figure 3A) and cockroaches than in blow flies and fruit flies. However, as is apparent from the scale bars on the confocal images, the sizes of cuticular caps were much smaller in blow flies and still smaller in Drosophila. Measurements of the long axes of the caps indicated that the mean sizes in blow flies was Group 3 6.17+/- 2.2 SD microns and Group 4 6.7 +/- 2.0 microns while the mean cap length in Drosophila was Group 3 4.1 +/- 0.6 microns and Group 4 4.1+/- 0.7 microns (total n = 78 sensilla measured in 12 groups, N = 18 animals). This difference was also apparent when all groups of campaniform sensilla were examined in blow flies and Drosophila. Figure 3B shows histograms that compares the number and sizes of the receptor caps of all sensilla groups in blow flies and Drosophila (total n = 162 sensilla in 30 groups, N = 18 animals). The number of sensilla (Fig 3B left) was very similar in both species but measurements of the cap long axes (Fig 3B right) showed that the cap size was significantly larger in blow flies than in Drosophila ($p < .01$ Student's t test).

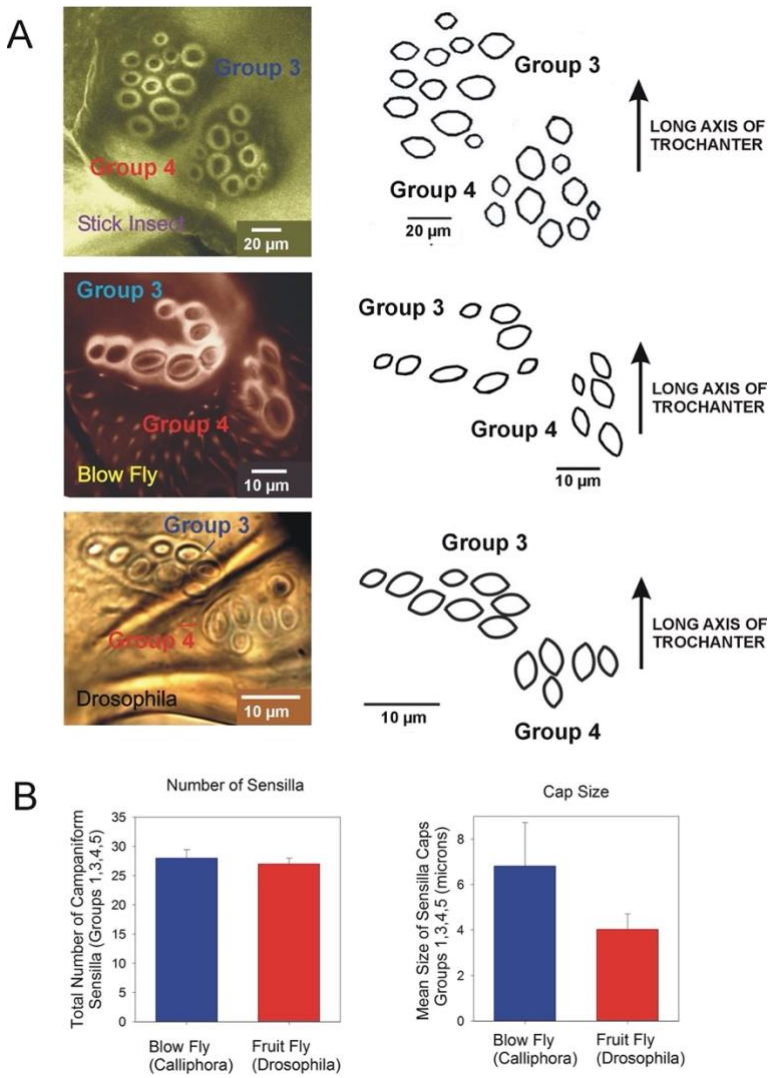


Figure 3: Structure of cuticular caps of Trochanteral Groups 3 and 4

A. Confocal images and outline drawing of cuticular caps of Trochanteral groups 3 and 4 in a stick insect (Carausius, upper) blow fly (Calliphora, middle) and fruit fly (Drosophila). The long axes of the large cuticular caps of the two groups are approximately mutually perpendicular in orientation in all three species. B. Plots of the numbers (left) and mean cap sizes (right) in all groups indicate that the number of sensilla is similar in blow flies and fruit flies, but the mean cap size is significantly smaller in Drosophila.

Diversity and species specificity in some groups of campaniform sensilla

Other groups of campaniform sensilla on the trochanter (Groups 1) and femur (Group 5) were similar in location but differed substantially in the size and arrangement of the cuticular caps. Group 1 could be identified by its location on the posterior side of the trochanter in all species but the number of caps in the group varied considerably. Figure 4 shows confocal projection images of Group 1 in a cockroach (Fig 4A), stick insect (Fig 4B), blow fly (Fig. 4C) and Drosophila (Fig 4D). Group 1 is large and shows a range of cap sizes in both cockroaches and stick insects but is greatly reduced in both blow flies and fruit flies, typically consisting of 3 sensilla. However, measurements of the cap length in flies indicated that there was a gradient of size in blow flies (Fig. 4E).

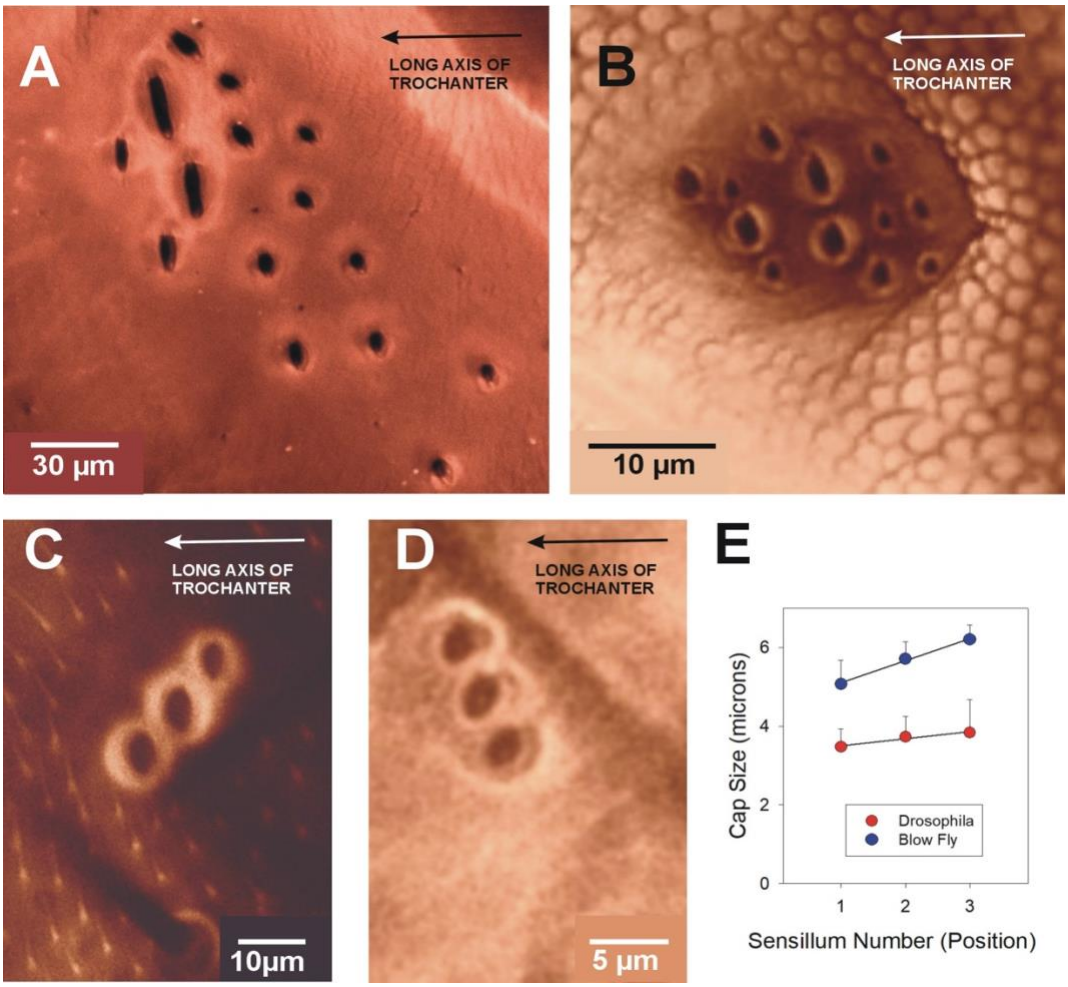


Figure 4: Species specificity in some groups of campaniform sensilla

A-C. Confocal projection images of posterior trochanteral group (Group 1) in a cockroach (A), stick insect (B), blow fly (C) and Drosophila (D). The number of sensilla varies widely between large insects (A,B) and flies (C,D). Group 1 in flies most often consisted of a row of three campaniform sensilla. E. Plot of cap sizes in blow flies and Drosophila, position which sensillum (1/2/3) is being referred to within group 1. The caps are larger in blow flies and show a steeper gradient in size.

Group 5 was found on the proximal femur but varied both in location and in the number of cuticular caps. Figure 5A is a confocal image of group 5 in a cockroach (Fig. 5A). Figures 5B and 5C show the caps of group 5 in blow flies and Drosophila. In cockroaches, Group 5 is located on the dorsal side of the femur and consists of 5-6 caps with a gradient of sizes. Group 5 is in a similar location in stick insects but is larger (16-17 caps) and contains caps of two orientations, as well sensilla with round caps (Zill et al., 2017). In flies Group 5 usually contains 11 cuticular caps in a consistent arrangement and overall gradients of cap size (Fig. 5D). Many of the caps are oval shaped and arranged in two rows at acute angles relative to the femoral axis (Fig. 5E). In addition, three of the sensilla, located on the distal end of the group, have large round caps. In flies and moths, Group 5 is located on the ventral surface (Gnatzy et al., 1987; Kent & Griffin, 1990), unlike all groups that contain multiple campaniform sensilla in cockroaches or stick insects (Fig. 5F). The location of the group and its cap orientation is therefore specialized and simple analysis of strain distribution suggests that the sensilla may be maximally sensitive to resisted contractions of the depressor muscle (Fig 5G). In flies, this may be associated with the large forces needed for the escape jump and flight initiation (Card & Dickinson, 2008).

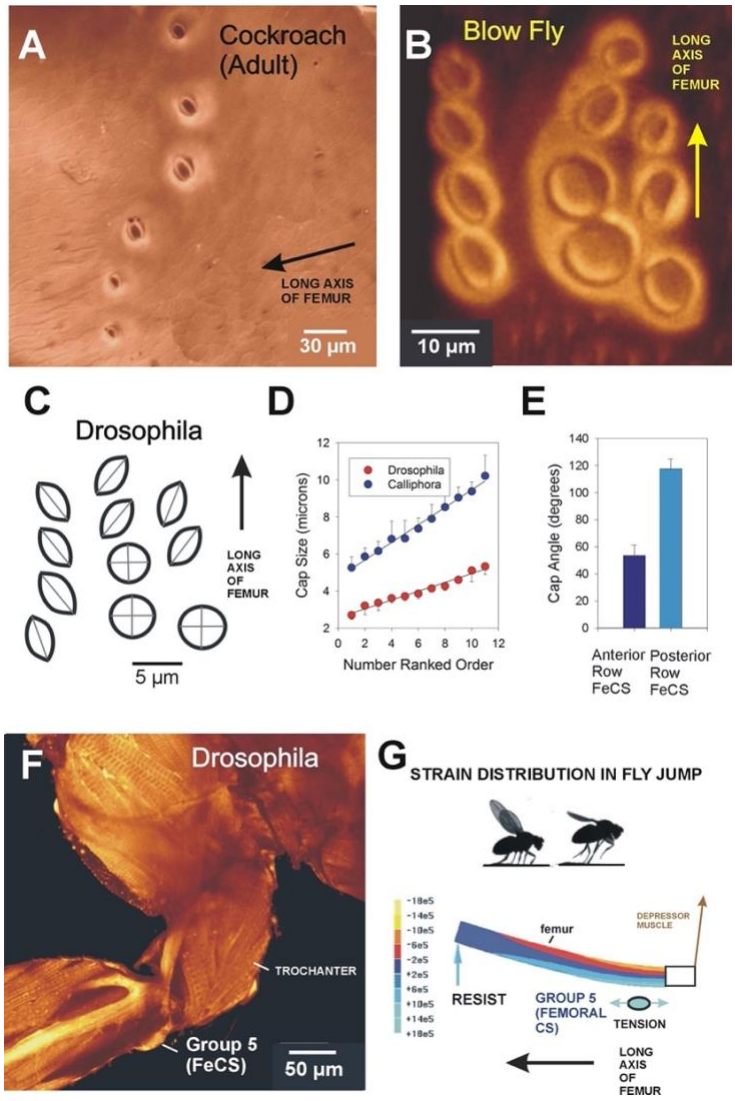


Figure 5: Femoral campaniform sensilla (Group 5)

A. Confocal Image of Group 5 (femoral campaniform sensilla) in a cockroach which is located on the dorsal side of the femur. B. Image of Group 5 in a blow fly - The group is particularly well developed in flies. C. Drawing of cuticular caps in Group 5 in Drosophila shows a similar pattern of ovoid and round caps as seen in blow flies. D. Size of cuticular caps in blow flies and Drosophila - The range and gradient of cap sizes in larger in blow flies. Rank order is the number of sensilla in the group from smallest to largest E. Cap angles - The ovoid sensilla are organized in two subgroups with consistently different cap orientations. F. Confocal image of a leg and leg

nerves in Drosophila infused with diI. The femoral campaniform sensilla (Group 5) are found on the ventral margin of the femur in flies. G. Diagram of strain distribution in a beam shows that the region of Group 5 sensilla would be under tension (exciting receptors oriented parallel to the segment long axis) when contraction of the depressor muscle is resisted, as occurs before a jump or take off in flight.

Comparison of number of campaniform sensilla in adult and juvenile cockroaches

The differences seen in sensilla of large insects (cockroaches and stick insects) and flies may be related to body mass. Campaniform sensilla were, therefore, also imaged in juvenile (first and second instar) cockroaches. In hemimetabolous insects both the size and mass of the animal increase in successive molts before reaching the adult stage, while flies are holometabolous and do not molt after eclosion. Figure 6A shows confocal images of the groups on the anterior surface of the trochanter in adult (left) and juvenile (second instar, right) cockroaches. Groups 3, 4 and 2 can be readily identified although the size of the trochanter is much larger in adult cockroaches. Figure 6B plots the numbers (left) and sizes (right) of cuticular caps in Groups 3 and 4 in preparations in which all sensilla were clearly visible. The caps of sensilla in Groups 3 and 4 increase both in number and in the length of the cap long axis, consistent with the general findings of Ridgel et al. (2003).

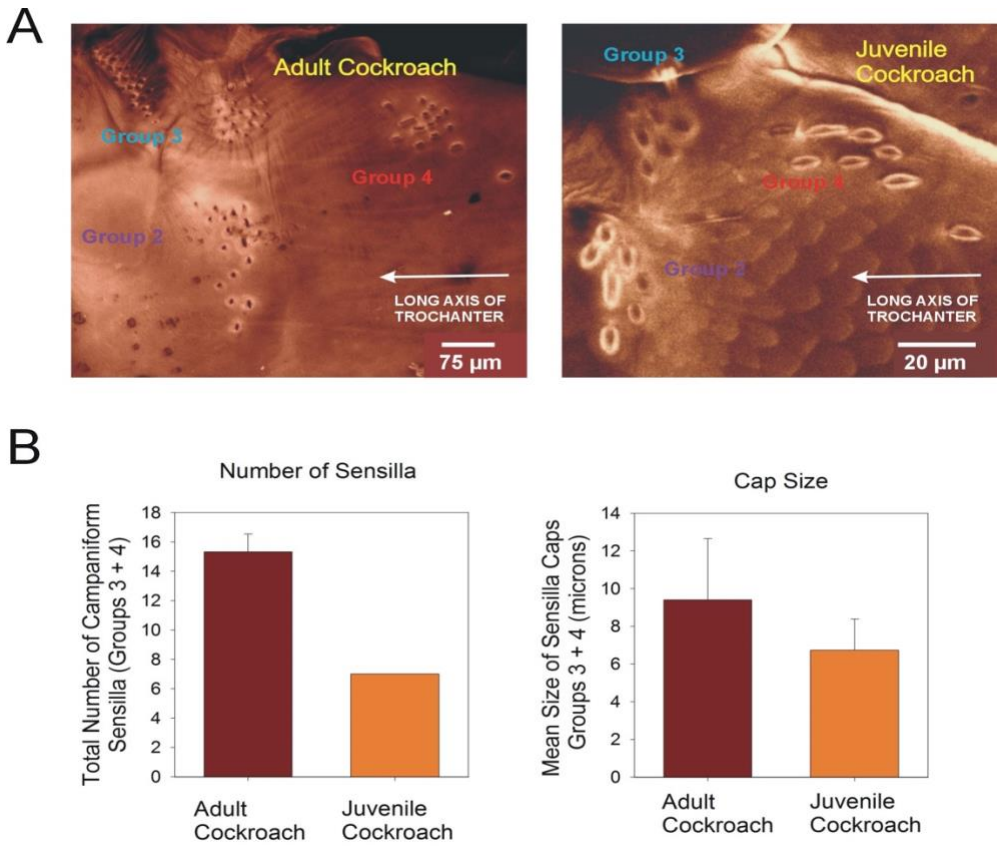


Figure 6: Comparison of caps of campaniform sensilla in adult and juvenile cockroaches

A. Confocal images of trochanteral campaniform sensilla in adult (left) and juvenile (second instar, right) cockroaches. The same groups can readily be identified at both stages of development. Groups 3 and 4 have mutually perpendicular cap orientations. B. Plots of the numbers (left) and mean sizes (right) in adults and juveniles. Both the number of sensilla and the diameters of long axes of the caps are smaller in juveniles.

Number of cuticular caps in trochanteral and femoral campaniform groups in all species: flies are similar to juvenile cockroaches

Figure 7A is a histogram which plots the mean number of campaniform sensilla in stick insects, adult and juvenile cockroaches, blow flies and fruit flies (n = 1999 sensilla in 71 groups, N = 56 animals). For each animal, data on groups which showed strong structural similarity

(Groups 3, 4) are shown on the left while the species specific groups (1, 2 and 5) are on the right. (Note: Data are plotted for stick insect middle legs but equivalent numbers of sensilla were found in hind legs (middle leg: total mean 66.6 +/- 10.4 SD; hind leg: mean 65.6 +/- 9.7 SD; total N =6) and in counts of individual trochanteral and femoral groups). The approximate weight of each animal is indicated above the histogram. Figure 7B shows the total number of campaniform sensilla in each animal (from the same data set) while Figure 7C plots number of sensilla found in Groups 3 and 4. All plots show that the largest numbers of sensilla are found in the animals with greatest size and weight (stick insect and cockroaches) although the numbers in specific groups vary between the two species. Smaller numbers of receptors are present in insects with small size and body weight but the mean numbers of receptors in blow flies and drosophila is quite similar despite a 30-fold difference in their weight. In addition, the numbers of sensilla overall and in Groups 3 and 4 specifically are similar in Drosophila and juvenile cockroaches. These data suggest that there is no reduction in force detection in fruit flies despite their minimal body weight.

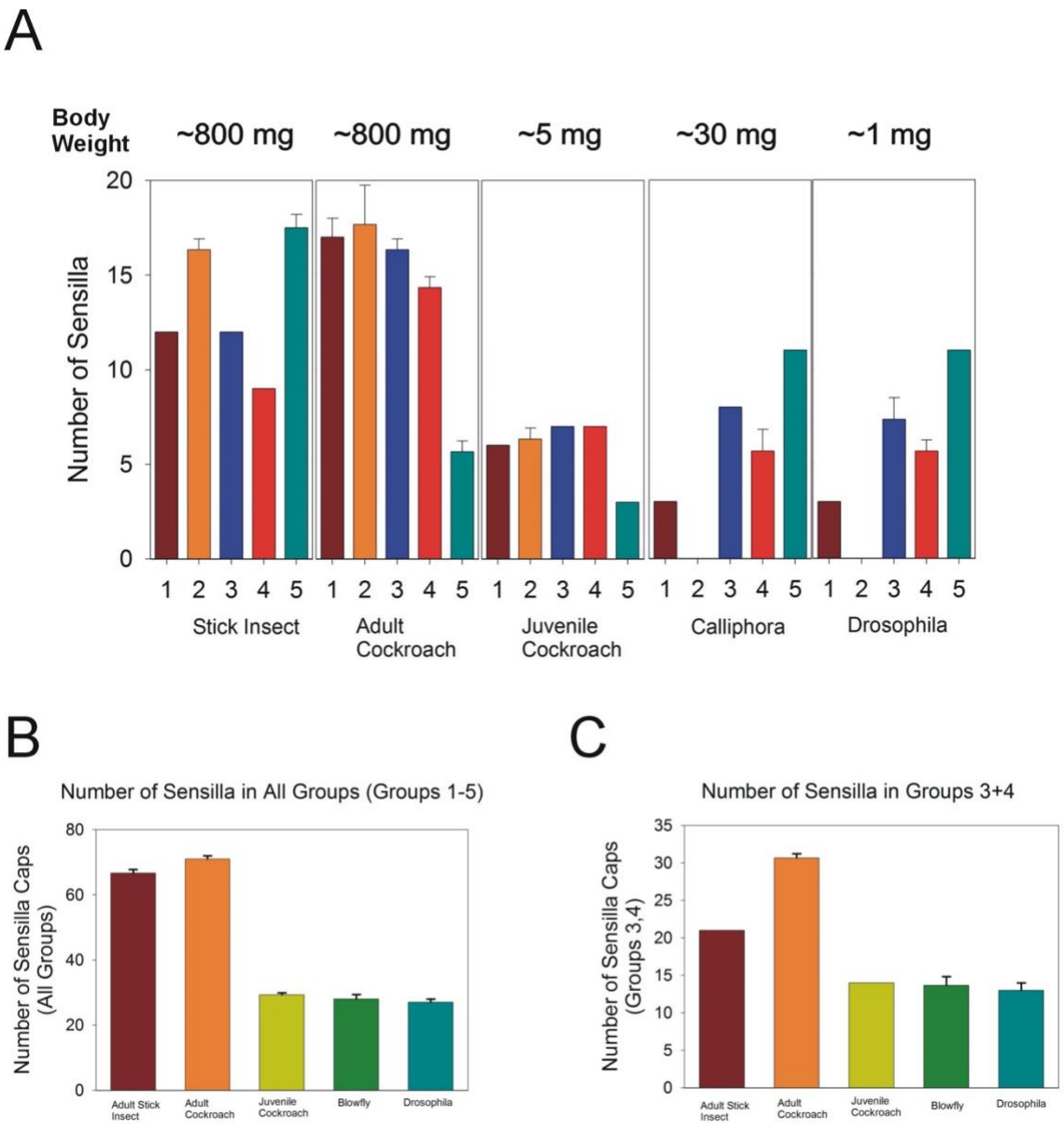


Figure 7: Comparison of the number of cuticular caps in different species

A. Histogram of the mean number of campaniform sensilla in all groups in stick insects, adult and juvenile cockroaches, blow flies and Drosophila (Group 2 is absent in flies). B. The total number of sensilla in all groups (1-5) in each species. C. Histogram of the mean number sensilla in Groups 3 and 4 in each species.

Gradients in sizes of cuticular caps and correlation with body weight

Previous studies support the idea that the range of forces detected by campaniform sensilla is related to the sizes of the cuticular caps (Zill et al., 2013). Figure 8A is a histogram that plots the mean sizes and standard deviations for the cuticular caps in all groups for all animals studied (same data set as Figure 6, n = 1999 sensilla in 71 groups, N = 56 animals). To indicate the distribution of sizes, the mean values of size of individual sensilla are shown as dots. The gradient of sizes of cuticular caps (indicated both by the distributions of cap size and the standard deviations from the mean) reflects the range of forces encountered by the animal: the gradients are broadest in animals with large body weights, smaller and similar in blow flies and juvenile cockroaches and smallest in Drosophila. Figure 8B is a plot (on a logarithmic scale) of the mean sizes of sensilla in each animal vs the body weight. There is a general correlation between the mean cap size and the weight of the animal. Linear regression analysis indicates a relationship ($r^2 = 0.86$) between mean cap size and body weight that could account for over 85% of the variance in the data.

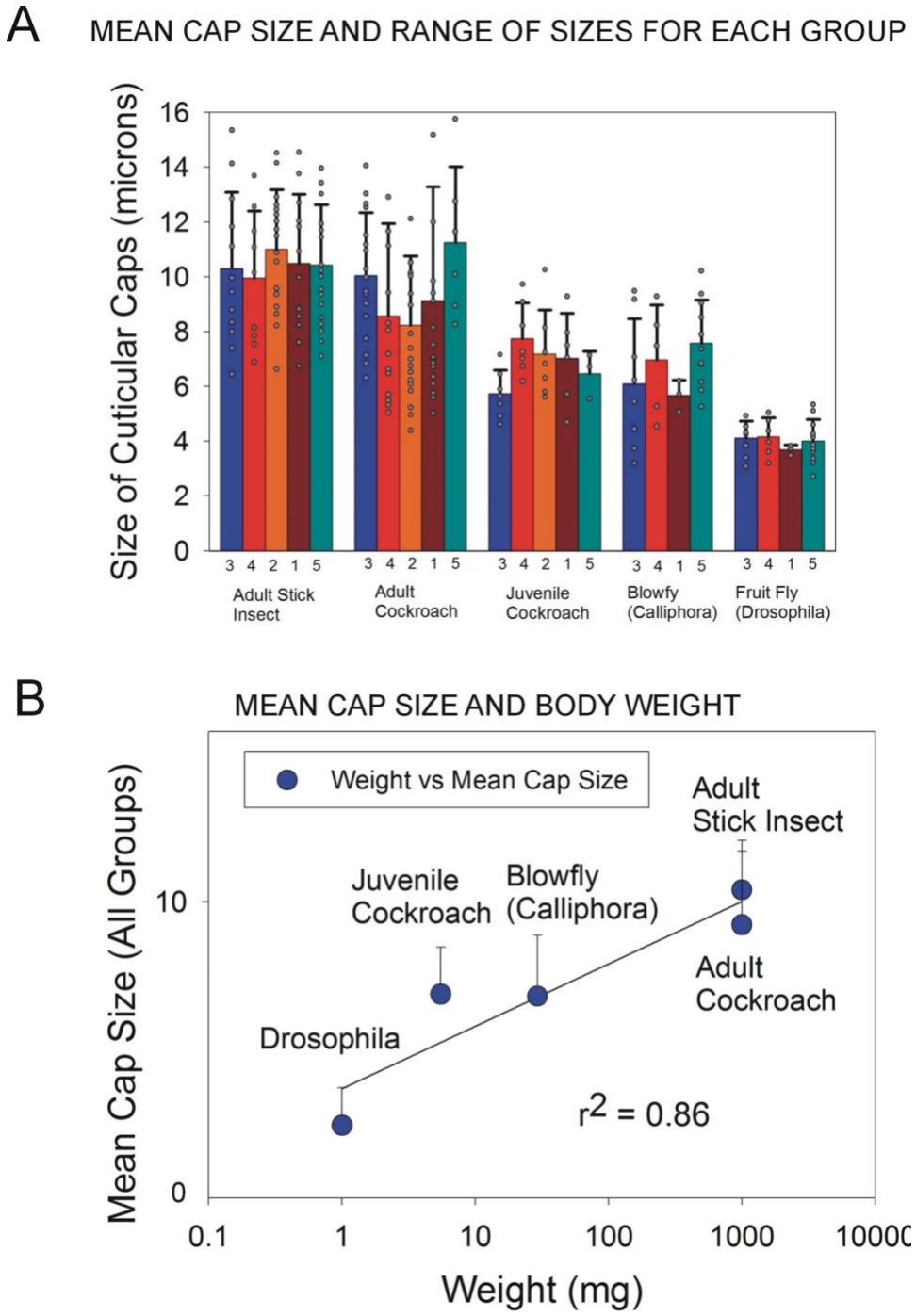


Figure 8: Gradients in sizes of cuticular caps and body weight

A. Histogram of mean sizes and standard deviations for all groups of campaniform sensilla in all species (measurements of n = 1999 sensilla in 71 preparations, N = 56 animals). To indicate the

distribution, the values of sensilla are shown as dots (see text). B. Plot of the mean cap size (all groups) for each species vs weight of animals ($r^2 = 0.86$).

Discussion

Similarity of groups of receptors based on morphology: trochanteral groups 3 and 4 are present in all species studied

Campaniform sensilla occur in isolation on the legs but are regularly found in groups that can be identified by their location (Pringle, 1938). The groups consist of discrete clusters, often located in pockets of cuticle that are thinner than the surrounding exoskeleton (Zill et al., 2000; Zill et al., 2012) and that potentially function as stress concentrators (Timoshenko & MacCullough, 1956). The present study has demonstrated that a number of groups on the trochanter can be found in similar locations in different species. Groups 3 and 4 located on the dorsal surface of the trochanter were consistently identified in every individual in all four species. Similar groups were shown to be present in almost all species of flies studied by Frantsevich and Gladun (2002). Two groups of sensilla are also found on the dorsal trochanter in moths (Manduca, (Kent & Griffin, 1990)) and similar receptors are present on the locust middle leg (Hustert et al., 1981) and cricket front leg (Nishino & Sakai, 1996), although they are somewhat spatially separated (as in cockroaches, (Zill et al., 2000)). The orientations of the ovate cuticular caps, which determine directional sensitivity, were also consistent in Groups 3 and 4 in all species: the cap long axes showed similar orientations within a group and the orientation of two groups were approximately mutually perpendicular. Similar arrangements have been documented in Groups 3 and 4 in the cranefly, *Tipula lunata*, and the muscid fly, *Mesembrina meridiana* (Frantsevich and Gladun (2002), Fig. 10).

Group 1 on the posterior trochanter was also found in all four species. Group 1 is well developed in stick insects and cockroaches but contains fewer receptors in flies. Posterior groups of campaniform sensilla are also found in many other flies (Frantsevich & Gladun, 2002), are also present in moths (Kent et al., 1996), locusts (Hustert et al., 1981) and crickets (Nishino & Sakai, 1996). The anterior trochanteral sensilla (Group 2) are present in stick insects and cockroaches, as well as other insects (moths, locusts and crickets see references above) but apparently absent in flies. In addition, a small group of campaniform sensilla, found to date only on the trochanter in stick insects, is associated with the mechanism of autotomy (leg loss) (Schindler, 1979). Therefore, some groups of campaniform sensilla appear to be strongly 'homologous' (similar in structure) while others are species specific.

Gradient in cap sizes as a factor in tuning sensory responses to body weight

Body weight is thought to be reflected, in part, in the number of leg sense organs that are activated. The present study has shown that the number of all campaniform sensilla in proximal leg segments reflects the mass and size of the animal and was significantly greater in larger insects (cockroaches and stick insects) than in flies, which are smaller and lighter (Figs 7). In addition, the number of receptors in flies was similar to that seen in juvenile (first and second instar) cockroaches. These findings support the idea body weight could be indicated by the number of sense organs that are activated, as is thought to occur in vertebrate Golgi tendon organs. However, morphological data indicated that blow flies and Drosophila had similar numbers of receptors despite a 30-fold difference in body weight, confirming previous findings for many species of flies (Frantsevich & Gladun, 2002).

Data from the present study showed that both the range and mean cap sizes were significantly larger in blow flies than in Drosophila, even though the number of cuticular caps

was approximately equivalent. Gradients in the sizes of the cuticular caps were found in almost all groups of campaniform sensilla on the proximal segments. In addition, comparison of cap sizes and body weight showed a strong correlation across species and in development. Gradients of cap size are also apparent in campaniform sensilla in other insects such as moths (Kent & Griffin, 1990), crickets (Nishino & Sakai, 1996) and locusts (Hustert et al., 1981) although measurements of cap diameters are not available.

Previous physiological data strongly support the idea that there is a correlation between cap size and the range of responses of campaniform sensilla. Recordings of the tibial and trochanteral sensilla showed that receptors with smaller extracellular potential have lower thresholds, responses ranges, and cap stimulation confirmed that those were receptors with smaller cuticular caps (Zill et al., 2011; Zill et al., 2013; Zill et al., 1999). The correlation between cap size and body weight is also supported by observations that the range of cap sizes increases in development of hemimetabolous insects (Biggin, 1981; Ridgel et al., 2003). Data from cockroaches and their molted exoskeletons suggest that the caps of existing receptors increase in size and new, smaller ones are added with each molt. Although the present study characterized receptors in very early instar and adult cockroaches, published data on the body weight of cockroaches in development (Gier, 1947) shows an exponential increase in weight late in development (ex. weight after five of 11 molts = ~50 mg, Adult = ~900 mg). During this time, there is a substantial increase in the size of the largest cuticular caps (Ridgel et al., 2003). In contrast, the number of sensilla shows a gradual increase throughout all successive molts. These changes can serve to increase the gradient and range of forces signaled by the existing receptors to match the growth of the animal, while adding small sensilla to retain sensitivities to low levels of force (i.e. the smaller the sensilla the lower level of force can be detected).

The difference in cap size is also reflected in the effects of campaniform sensilla on motor outputs. Studies that used mechanical stimulation of individual cuticular caps showed that reflex activation of motor activity can regularly be elicited by stimulation of large cuticular caps but rarely by sensilla with smaller caps ((Zill et al., 2011; Zill & Moran, 1981a); personal observation). Effects on motor output, tested by using waveforms of joint torques, are much larger in ranges in which large sensory units are recruited (Zill et al., 2018). However, quantitative relationships between strain sensitivity and motor effects have not been established, in part, because the effects are state dependent.

Specific data on the potential correlation between sizes of cuticular caps and the somata of sensory neurons is not available although differences in cell body size within groups of campaniform sensilla are apparent (Figure 2B). Data from other arthropods indicate that the relationship may be complex: spider lyriform organs are composed of parallel slits of varying size (Barth & Bohnenberger, 1978; Schaber et al., 2012). However, the sizes of the sensory somata only match the gradient of some, but not all slits (Fabian & Seyfarth, 1997).

Sensing body weight is an active process that is dependent upon tensions in antigravity muscles

Previous studies using physiological recordings (Zill et al., 1999) and finite element modeling (Flannigan, 1998; Kaliyamoorthy, 2003) indicate that groups 3 and 4 are positioned on the dorsal trochanter to maximally encode strains produced by forces of the muscles that depress and levate (lift) the trochanter (Zill et al., 2012). In many insects, the depressor and levator are among the largest intrinsic muscles of the leg (Schmitz, 1986, 1993; von Twickel et al., 2019) and the trochanteral sensilla have been shown to have strong effects in modulating depressor activities (Höltje & Hustert, 2003; Pearson, 1972; Zill et al., 2012). The depressor also has been

demonstrated to be activated by forces that increase the effects of body weight in freely moving animals (Quimby et al., 2006; Rosenbaum et al., 2010) and, when cockroaches climb over a block, the leg is moved to optimize the mechanical effectiveness of the depressor (Watson et al., 2002). Furthermore, in an upright posture, group 4 sensilla can encode decreases in the effects of gravity, as could occur during leg slipping (Kaliyamoorthy et al., 2006; Keller et al., 2007). In contrast, Groups 1 and 2 are positioned to detect forces acting in an anterior and posterior direction, perpendicular to the major leg plane (Kaliyamoorthy, 2003; Schmitz, 1993). Posterior forces strongly activate retractor muscles in stick insects (Borgmann et al., 2011; Haberkorn et al., 2019; Schmitz, 1993) and signals from these groups may also contribute to changes in motor activities during climbing or ascending a ramp (Dallmann et al., 2016, 2019; Kaliyamoorthy, 2003).

Constancy and variability in organization of force detection

While similar groups of campaniform sensilla could regularly be identified in different animals, some measure of variability occurred in the number, location and sizes of campaniform sensilla. In Pringle's original description of cockroach campaniform sensilla (Pringle, 1938), the numbers of sensilla on the right and left legs were not invariably the same (usually varying by one or two sensilla). Variations in sensillum number and arrangement can occur in hemimetabolous insects if legs are regenerated after being damaged in molting or following autotomy in resisting a predator (Biggin, 1981; Lakes & Mücke, 1989; Maginnis, 2008; Maruzzo et al., 2005). Holometabolous insects, such as flies, do not molt after emergence but residual leg damage can occur in eclosion (Frantsevich et al., 2017), although the effects on sensory structures have not been determined. In the present study, small variations in the number of sensilla occurred in both blow flies and Drosophila, in contrast to the findings of Frantsevich and

Gladun (2002) who studied 205 species of flies of a range of body lengths (2.5 – 20 mm) and found the same number of sensilla in groups of all Cyclorrhapha. One factor that could contribute to differences in determining the number of receptors is the sizes of the caps of the smallest sensilla, which can be difficult to discern from broken seta McIndoo (1914) discussed by Pringle (1938). We found that the smallest caps could only be confirmed as campaniform sensilla by serial optical sections in confocal imaging and, in a comprehensive study of the anatomy of sensilla on all legs in Drosophila, Dinges et al. (2019) (also Dinges, submitted) have also found variation in the number and locations of receptors in Drosophila using scanning electron microscopy.

We also observed variability in the occurrence of 'isolated' sensilla. Groups of single or pairs of sensilla have been consistently identified in flies (Gnatzy et al., 1987) and locusts (Burrows & Pflüger, 1988). It is important to note that campaniform sensilla can potentially have both specific and non-specific effects on motor outputs (Bidaye et al., 2018). Specific effects include gating of context-dependent behaviors, such as the inhibition of searching movements by substrate contact in the stick insect (Berg et al., 2015), while non-specific effects include the modulation of motor response strength by inputs to octopaminergic dorsal unpaired median neurons (Stolz et al., 2019). In insects, the arrangement in groups appears to be more consistent in proximal rather than distal segments, associated with the strong effects of trochanteral and femoral sensilla on leg muscles (Akay et al., 2004). In contrast, the number and arrangement of the tibial sensilla is variable in some insects: in the stick insect tibia, two subgroups (Groups 6A, 6B) were identified based upon activity that could be recorded and associated with specific receptors by cap stimulation and ablation (Zill et al., 2011). However, subsequent studies have identified other sensilla in the proximal tibia, often not in close proximity to the receptors that

were identified physiologically (Haberkorn et al., 2019; Strauss, 2020). Campaniform sensilla on the tarsi of insects also show considerable interspecies variability (Joel et al., 2018). This variability may reflect the fact that campaniform sensilla can have mass effects as a population and contribute to the general activation of the system following leg contact. This function could be fulfilled both by sensilla organized as groups and by isolated receptors.

Specializations of the femoral campaniform sensilla: role in jumping for flight take off in flies

The femoral campaniform sensilla showed the largest differences in location and in the number of sensilla. Group 5 was relatively small in cockroaches but large in stick insects although gradients of cap size occurred in both groups. In both species, the group is located on the posterior surface of the femur but the receptors show differences in responses that are associated with cap orientation (uniform in cockroaches, diverse including round caps in stick insects) and the mobility of the trochanter-femur joint (highly mobile in cockroaches but fused in stick insects (Zill et al., 2017). The femoral campaniform sensilla in stick insects (but not cockroaches) encode forces in the plane of the coxo-trochanteral joint and have broad effects in the activation of muscle synergies (Akay et al., 2004; Zill et al., 2017).

In contrast, the femoral campaniform sensilla of flies are found on the ventral surface of the femur where they form a prominent elevation on the cuticle near its joint with the trochanter (Stern, 2003). In both blow flies and Drosophila, the caps form a distinct pattern with rows (columns) of oval caps and large centrally located receptors with round caps (Gnatzy et al., 1987; Merritt & Murphey, 1992). A femoral group, containing sensilla with round caps, is also found on the ventral proximal femur in moths (Kent & Griffin, 1990). The location and cap orientations of the sensilla suggest that they could encode depressor forces, both during walking and in the

jumping which precedes initiation of flight (Card & Dickinson, 2008; Hammond & O'Shea, 2007; Soler et al., 2004; Trimarchi & Schneiderman, 1995). The jump is generated by the contraction of depressor muscles in all legs and the femoral sensilla in flies and moths are the only leg campaniform sensilla found to project intersegmentally (Kent et al., 1996; Merritt & Murphey, 1992; Tsubouchi et al., 2017) while femoral sensilla of cockroaches, stick insects and locusts are restricted to the single corresponding segmental thoracic ganglion (Haberkorn et al., 2019; Schmitz et al., 1991). The complex arrangement of receptors in the femoral sensilla may also contribute to the behavioral flexibility in flight initiation and escape jumping (Card & Dickinson, 2008).

Limitations to this study

The number of species we examined was limited, although it included insects in which sensory activities have been recorded physiologically and the control of posture and locomotion has been extensively studied (Bidaye et al., 2018). Further studies are also needed to see if gradients of cap sizes are tuned to body weight in other species of Cyclorrhaphid flies, which previous studies have shown to have similar numbers of campaniform sensilla. As noted above, the correlation of cuticular cap size with physiological properties needs to be confirmed, particularly in sensilla with round cuticular caps. However, the presence of round caps does not necessarily imply that receptors are omnidirectional (as in insect wings) as some of the tibial campaniform sensilla in stick insects (Group 6B) have round cuticular caps (but oval collars) and show directionality in responses to cuticular strains (Zill et al., 2011). In sum, the findings of the present study support the idea that the gradients in sizes of cuticular caps could serve as a mechanism for setting receptor sensitivity to body weight, although additional data are needed to correlate the morphology and sensory processing of forces encountered by the animal.

The size principle and sensory transduction of body weight

The present study has shown that similar groups of campaniform sensilla can be identified in a number of insect species. The arrangement of sensilla in groups (in contrast to isolated receptors) has the advantages that 1) sensilla in a particular location with similar cap orientations respond to the same direction of strains and 2) sensilla of different cap sizes respond to different ranges of strain, and therefore show range fractionation to forces. The gradients of size of the cuticular caps can be considered as components parallel to the size principle of organization of motor systems within the central nervous system (Azevedo, et al., in press). In many animals, motor neurons are activated in a graded order according to their size and magnitude of force output, with small units being recruited first. In sensory systems, a corresponding relationship describes the sensitivity of sensory neurons (Weber-Fechner Law, Johnson et al., (2002)). However, the gradients of cap size may also represent a matching of sensory transduction to the range of force output. Previous experiments on reflexes of campaniform sensilla indicate that receptors with large cuticular caps are the most effective in activating motor neurons. Further studies are needed to test whether there is a consistent relationship between the response range of sense organs and effects on motor output. However, motor responses must be adjusted to body weight which is greater in larger animals and increases in development of individuals. The gradients in mechanotransduction may represent components of emergent, graded processes that do not require the precise calculation of body weight. Similar mechanisms may be present in Golgi tendon organs, which show gradients in size and range fractionation in force detection. Matching properties of sensory inputs to motor outputs could be utilized in both invertebrate and vertebrate systems to similar advantage.

USING CONFOCAL MICROSCOPY TO INVESTIGATE THE NEURAL MECHANISMS UNDERLYING RODENT BEHAVIOR

The studies in this dissertation focus upon the neural mechanisms underlying behaviors in insects and rodents. The first study examined correlations of structure and function in sensation. Insects have a simple nervous system when compared to rodents and human but both invertebrates and vertebrates must resolve the problem of adjusting motor behaviors to the weight of the animal. We utilized confocal microscopy to characterize structures that mediate mechanotransduction in receptors (campaniform sensilla) encoding forces in the legs and showed that detection of body weight was reflected in both the number and range of sizes of the receptors. In the second study, we examine the how non-neuronal glia called astrocytes and microglia contribute to the behaviors of rodents and the effects of alcohol on nervous system function. In the following, we show that 1) behavioral tests have documented removal of three factors involved in astrocyte activation effect anxiety and reward related behavior and 2) confocal imaging has shown that removal of this astrocyte activation pathway alters the expression of molecules that may mediate anxiety and reward motivated behavior. In sum, these studies have shown that behaviors in diverse species depend upon specific nervous system functions and that these mechanisms can be elucidated by confocal microscopy.

ABSTRACT

Binge alcohol/ethanol (EtOH) consumption peaks in adolescence and the early twenties, coinciding with a period of increased risky decision making. The binge pattern of exposure also increases the likelihood of developing an alcohol use disorder later in life. However, the underlying cellular mechanisms that drive these changes are not well understood. Recent work has implicated neuroimmune glial processes in the development of long-term alcohol-induced changes in brain function; however, there remain significant gaps in knowledge regarding the relationship between neuroimmune activation and the development of sensitivity to addictive substances. Previous work in our laboratory and others has demonstrated that microglia and astrocytes display stereotypical reactive phenotypes in response to repeated ethanol exposure. Of particular interest are interleukin-1 alpha (IL-1a), tumor necrosis factor-alpha (TNF-a), and complement component 1q (C1q); signaling proteins released during microglial activation that induce astrocyte reactivity. Our work investigates the role of this pathway in reward sensitivity, anxiety-like behavior as well as changes in neuroinflammatory cell expression following repeated adolescent EtOH exposure.

CHAPTER 3

INTRODUCTION

Alcohol Use And Abuse In Perspective

Alcohol use and abuse is highly prevalent within the United States. According to the Substance Abuse and Mental Health Services Administration's (SAMHSA) 2020 National Survey on Drug Use and Health, 162.5 million people aged 12 and older have consumed some form of substance within the last calendar year (Substance Abuse and Mental Health Services Administration, 2021). Further, they report in 2020 that 138.5 million people aged 12 and older used alcohol with 61.6 million of those being past month binge drinkers (Substance Abuse and Mental Health Services Administration, 2021). In their 2019 report, 139.7 million people aged 12 and older used alcohol, while 65.8 million of those were classified as binge drinkers (Substance Abuse and Mental Health Services Administration, 2020). These numbers are alarming and highlight the prevalence of alcohol use. These reports further highlight alcohol use among adolescents. This is particularly important as adolescence is a period that is hallmarked by increased risky decision making, increased freedom as well as brain maturation (Spear, 2000). The SAMSHA's 2020 report notes among binge drinkers, those who are aged 18-25 had the highest incidence of binge drinking (10.5 million), while in 2019, 11.9 million 18-25 year old's were classified as binge drinkers (5 or more drinks for males, 4 or more drinks for females in 2 hours) (Substance Abuse and Mental Health Services Administration, 2020, 2021). In addition to binge drinking these reports note that 6 million (2020) and 7 million (2019) of those aged 12-to-20 used alcohol in the last month (Substance Abuse and Mental Health Services Administration, 2020, 2021). However, it is important to note these numbers have decreased from 2002 where 10.7 million 12-to-20-year old's used alcohol in the past month (Substance Abuse and Mental

Health Services Administration, 2020). Further, as 2022 reports are being formulated it should be noted that the COVID-19 pandemic may have altered these numbers either decreasing or increasing the number of adolescents consuming alcohol and binge drinking. Though the statistics are not currently available, it will be interesting to see how the recent pandemic may have affected the trends in adolescent drinking. Additionally, though recent statistics show a decline in overall past month alcohol use, these numbers are still considerably high and are still a major cause for concern.

High levels of alcohol consumption are not solely linked to the United States, the World Health Organization published a report in 2018 highlighting the alcohol use worldwide. In 2016, 2.3 billion people worldwide were considered current drinkers, with many located within Europe, Western Pacific, and the Americas (World Health Organzation, 2018). Additionally, in 2018, 155 million adolescents (15-19 year-olds) worldwide were considered to be current drinkers, with prevalence of adolescent drinking in Europe and the Americas at 43.8% and 38.2%, respectively (World Health Organzation, 2018). These statistics point to a high level of alcohol consumption in both adults and adolescents worldwide, revealing an alarming trend of ongoing alcohol use. Further, they point to a need for studies which investigate the consequences of alcohol use and abuse in adolescence and adulthood.

Adolescent Alcohol Use: A Human Perspective

Studies show alcohol can act as a depressant or as a stimulant on the central nervous system (CNS), and can cause alterations in memory, delay reaction times and lead to difficulty walking (National Institue on Alcohol Abuse and Alcoholism, 2004; Rao & Topiwala, 2020). There have been several studies investigating the behavioral, cellular and molecular effects of adolescent alcohol use on the brain. As noted above, adolescent alcohol use is prevalent

particularly in the United States. Introduction of alcohol to a developing adolescent brain can lead to several detrimental and long-term effects. Interestingly, adolescents are more sensitive to the rewarding effects of alcohol and are less sensitive to the motor impairing effects of alcohol (Spear, 2014). An elegant schematic found in Salmanzadeh et al. (2020), shows many of the underlying effects of alcohol exposure in humans. One of the effects of adolescent alcohol exposure that is highlighted is an increase in anxiety in adulthood (Jeanblanc, 2015; Salmanzadeh et al., 2020). Interestingly, a strong correlation between adolescent alcohol exposure and increased social anxiety in adulthood has been found in humans (Crews et al., 2016). This finding shows lasting behavioral changes that persist into adulthood. Also noted by Salmanzadeh et al. (2020) adolescent alcohol exposure can increase the likelihood of using other substances of abuse particularly in adulthood. Grant and Dawson (1997) note that adolescent alcohol, particularly age of first use is a potent predictor for future use and abuse of alcohol. Further, Grant et al. (2006) found that adolescent alcohol use influences adult drug use in twins, showing a unique genetic component that can influence alcohol use and future drug use. A five year longitudinal study by Jackson et al. (2002) reveals an association between smoking and alcohol use in adolescents, noting smoking onset being higher for those with previous alcohol use. This study further revealed that the persistence of drinking can be predicted by year one tobacco use, while year one alcohol use predicted smoking persistence (Jackson et al., 2002). In short Jackson et al. (2002) shows continued alcohol use can be predicted by prior tobacco use and prior alcohol use can predict continued tobacco use, revealing a unique relationship between prior/ future alcohol and tobacco use. In addition to behavioral changes, studies reveal adolescent alcohol exposure can lead to changes in the prefrontal cortex (PFC) specifically in PFC volume (Salmanzadeh et al., 2020). This finding is further confirmed in a study by De Bellis et al.

(2005), which found adolescents and young adults with alcohol use disorders (AUD) onset in adolescence had smaller PFC volume compared to healthy subjects. This result is particularly important as the PFC is the brains hub for decision making. Changes in its volume may be correlated with decreased impulse control and increased risky decision making. In addition to changes in PFC volume, Salmazadeh et al. (2020) reveals adolescent alcohol use can change hippocampal volume. The hippocampus is the brains hub for learning and memory and is highly interconnected with other brain regions. A previous study revealed adolescents with AUD have smaller left hippocampal volume compared to healthy controls (Nagel et al., 2005). Similar to the PFC, decreases in hippocampal volume can lead to detrimental effects in learning and memory. This can become increasingly problematic as adolescents enter adulthood. Adolescent alcohol induced alterations to brain structure are not limited to the PFC and hippocampus, as gray matter volume can decrease while matter volume can increase across brain regions (Lees et al., 2020). For further discussion on these findings see Lees et al. (2020). Ultimately, adolescent alcohol use can have broad acute and long-term effects on behavior as well as structural and functional deficits in key areas of the brain. It is important to note that these studies reveal important correlative implications following adolescent intermittent ethanol exposure (AIE); however, establishing a causative relationship is more difficult. This is where animal studies are favored allowing the collection of baseline data that can inform scientists about whether decreased brain volume is a driver or a consequence of excessive drinking. Further, there are several limitations to human studies including ethical concerns, challenges with retaining subjects for long-term studies, and high variability in the physiological characteristics and mental health of study participants. Rodent models can provide complementary, unique insight into the effects of AIE while controlling for environment, health, social and external influences. Further,

rodent models offer the ability to study genetic drivers and mechanisms of disease whilst also allowing for tissue collection at multiple time points and assessment of the long-term, chronic effects of alcohol exposure, studies that are difficult in humans.

Adolescent Alcohol Use: Animal Studies

Many of the cognitive, behavioral, and neuronal changes taking place following alcohol exposure found in humans have been and/ or are currently being investigated in animal models. These animal models play a critical role in increasing our understanding of the cognitive and behavioral changes following adolescent alcohol exposure. Within the last 25 years the number of adolescent alcohol exposure studies have significantly increased. There are a number of methods to explore adolescent alcohol exposure, many of these methodologies fall under the adolescent intermittent ethanol (AIE) exposure paradigm or a chronic intermittent ethanol (CIE) exposure paradigm commonly using rats or mice. These studies often use animals in early adolescence post-natal day (PND 25-30) and often continues throughout middle to late adolescence (PND 35-42) (Figure 9) (Flurkey et al., 2007). Using Wistar rats who were exposed to 3g/kg of 25% EtOH (i.p.) intermittently between PND 25-38, Pascual et al. (2007) found increased levels of inflammatory mediator cyclooxygenase-2 (COX-2) and inducible nitric oxide (iNOS) in the hippocampus, neocortex and cerebellum. The results also show deficits in multiple behavioral tasks, using rotarod, which assesses deficits in gait and motor coordination at PND 39 (24 hours after last dose) in adolescents and PND 60 in adults, finding that EtOH exposed rats spent less time on the rotarod on day 6 of testing compared to other groups (Pascual et al., 2007). Further, they found that the EtOH exposed group preformed worse during object recognition at PND 39 and PND 60, a test which assesses non-spatial working memory, and had lower scores during beam walking assessments, which investigates motor coordination and balance at the

same time points (Pascual et al., 2007). This study by Pascual et al. (2007) revealed that EtOH induced cell death was correlated with acute and long-term changes in behavior which persisted into adulthood. An AIE study using Wistar rats intermittently exposed 5g/kg of EtOH (i.g.) 3 times-a-day between PND 28-53 investigated the effects of prior alcohol exposure on reward probability in adulthood, finding that AIE exposed rats had greater preference for risky reward when delivered with low probability in both ascending and descending trials (Boutros et al., 2015). Ultimately, the authors found that AIE exposed rats prefer a reward when the reward is paired with low risk. It is important to note that this study uses a paradigm where alcohol is given multiple times a day, therefore these results may vary from the traditional "single" dose AIE/CIE paradigms. Additionally, 5.0 g/kg via gavage brings the blood ethanol concentration to 170-190 milligrams per deciliter (170-190 mg/dl), which is considered binge-level; however, EtOH metabolizes relatively quickly in rats (Dasgupta, 2017; Livy et al., 2003). It is important to note that route of administration and species can affect the results of the study and level of intoxication as mouse oral gavage (i.g.) and intraperitoneal (i.p.) injection lead to a rapid rise in intoxication and elimination while rat gavage and intraperitoneal injections show gradual levels of intoxication while EtOH stays in their blood streams longer (Livy et al., 2003). Further, i.p. injections lead to the highest blood alcohol concentrations (BAC); however, show a rapid decline after reaching its peak, though gavage has lower initial BAC's it has a gradual decline over time (Livy et al., 2003). Despite a high alcohol dosing paradigm used in the study by Dasgupta (2017), their work reveals an important link between adolescent alcohol exposure and risky decision making related to reward. This is important for understanding the relationship between early alcohol exposure and the consequences of adolescent alcohol use later in life. In a mouse study of AIE, DBA/2J mice were intermittently exposed to 4g/kg of 25% EtOH (i.g.) between

PND 29-42, the results show 24 hours following the last dose the EtOH exposed mice have increased ambulatory time and distance traveled. Moreover, when these tests were run in adulthood the results show females exposed to EtOH had increased locomotor activity (Wolstenholme et al., 2017). This study further revealed decreased object recognition was found after a short delay (5 minutes) in the EtOH exposed male and females with females also showing a decrease in working memory after a long delay (1 hour) (Wolstenholme et al., 2017). The results of this study show intermittent adolescent alcohol exposure causes short- and long-term changes in locomotor behavior (i.e. movement, freezing, distance traveled) and working memory (i.e. short-and long-term memory association with an object). Assessing time in the light of the light-dark box is commonly used to investigate development of anxiety-like behavior, mice who are anxious tend to avoid brightly illuminated open spaces, therefore, a mouse that spends little time in the light is typically deemed anxious (La-Vu et al., 2020). Interestingly, the previous study by Wolstenholme and colleagues did not find any significant difference for time in the light between their groups, suggesting that the development of anxiety-like behavior may be dose dependent. Though their study did not find any significant differences in anxiety-like behavior between the AIE and control groups, there are studies using rats that have. An AIE study using Sprague-Dawley rats exposed to 2g/kg of 25% EtOH (i.p.) shows EtOH exposed rats spent less time in the light 24 hrs. after the last dose with anxiety-like behavior persisting into adulthood (Pandey et al., 2015). Another AIE study that exposed Wistar rats to 3g/kg of 20% EtOH (i.g.) for 3 consecutive days show no change in anxiety like behavior compared to controls in the elevated plus maze (EPM) (Torcaso et al., 2017). Variability between anxiety outcomes in the various animals models and across laboratories suggests that the behavioral effects of AIE may be dependent on choice of animal and exposure paradigm. These differences show the unique

and broad effects of alcohol exposure and withdrawal, and highlight the need for continued AIE mouse and rat studies to determine why these differences exist. The study by Pandey et al. (2015) further assessed the effects of AIE in rats on synaptic remodeling showing decreases in brain derived neurotrophic factor (BDNF) and Arc protein mRNA in the central nucleus of the amygdala (CeA). Further, they found decreases in dendritic spine density in the CeA and increases histone deacetylases (HDAC) in the amygdala leading to decreases in histone H3-K9 acetylation in the CeA and medial amygdala (MeA). These findings note the effects of AIE on production of factors such as BDNF, Arc protein, dendritic spines, and histones which play roles in long-term potentiation (LTP), neuronal survival and differentiation, synaptic transmission/plasticity, and gene expression (Martire & Banaszynski, 2020; Miranda et al., 2019; Nikolaienko et al., 2018; Runge et al., 2020). Changes in these factors and others may be the underlying drivers of long-term changes in anxiety-like behavior, learning and memory formation, as well as cellular/ molecular function (personal observation). A recent study by Risher et al. (2015) also investigates the effects of AIE on long term potentiation (LTP), synaptic plasticity and structure as well as dendritic spine morphology in male Sprague-Dawley rats who were intermittently exposed to 5g/kg of 35% EtOH (i.g.). This study reveals a lowered threshold for LTP induction that correlated with an increase in immature dendritic spines in adulthood (M.-L. Risher et al., 2015). To further characterize changes induced by AIE a second study by Risher and colleagues (2015), using the same AIE paradigm, showed increased synapse formation (synaptogenesis) as well as upregulation of thrombospondin 2 and 4 which are astrocyte secreted proteins that are critically involved in developmental and adult synaptogenesis (Eroglu et al., 2009). This upregulation of synaptogenic factors points to synaptic remodeling following AIE. This is important as synaptic remodeling can cause fluctuations of individual synapses as well as

alter/ disrupt established and developing networks, and may lead to changes in memory formation (Ziv & Brenner, 2018). In an addicted brain these newly formed synapses become mature and can integrate into the existing circuitry, increasing receptor expression and can drive the motivation to seek the drug of abuse particularly during the withdrawal period (Russo et al., 2010). However, it is important to note that the totality of the effects of synaptic remodeling particularly in established networks are yet to be fully elucidated. Also noted in this study are the changes to astrocytes, which are one of the most abundant glial cells in the CNS (M. L. Risher et al., 2015). Their study shows alterations to astrocyte structure through increased astrocyte volume and branch length (M. L. Risher et al., 2015). Ultimately, these studies further the understanding of the relationship between early alcohol exposure and the consequences later in life, and shed light on potential cellular and molecular mechanisms driving these long-term changes.

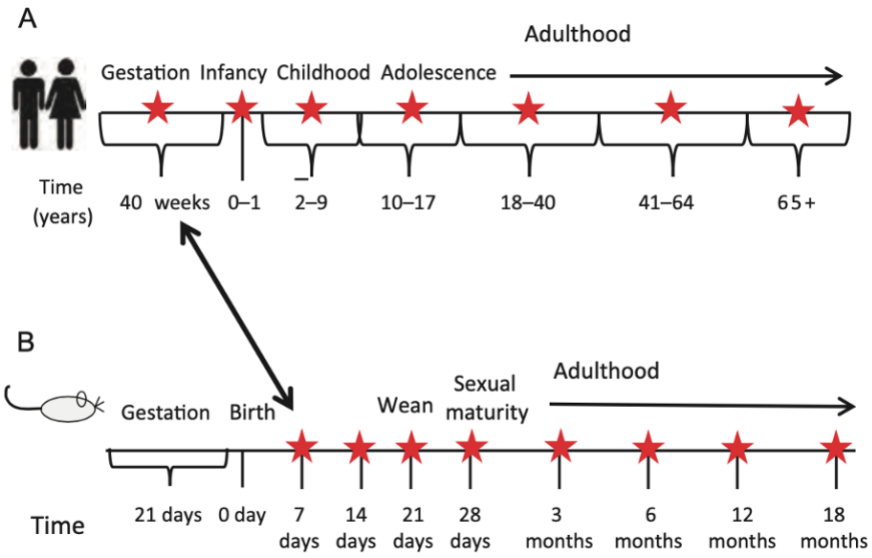


Figure 9: Mouse age and human age equivalences

This figure is from a blog post from Jackson laboratory entitled “When are mice considered old” the data for this figure is from Beamish et al. (2017).

Cellular And Molecular Perspective: What May Be The Underlying Mechanisms Driving Cognitive And Behavioral Changes Following Alcohol Exposure

The central nervous system (CNS) has two types of cells: glia and neurons. Neurons form the functional aspect of the central nervous system. They transmit information through the CNS via action potentials (electrical signals), and can be classified as sensory, motor, or interneurons (*Overview of neuron structure and function* 2011). While glia, which are vital for proper neuronal function have classically been referred to as support cells and historically overlooked (Rye et al., 2016). Ramon & Cajal and Rudolf Virchow were among the first to identify glial cells. There are several types of glial cells in the CNS including: astrocytes, microglia and oligodendrocytes (Jessen, 2004). Oligodendrocytes are a highly specialized glial cell that produce myelin, and are responsible for axonal myelination which increases the rate of signal transmission between neurons (Bradl & Lassmann, 2010; Jessen, 2004; Susuki, 2010). Microglia are the resident macrophages in the CNS and account for 10% of the cells in the CNS (Colonna & Butovsky, 2017). They play a number of roles including support for neurogenesis, influencing cell death or survival, and CNS repair following injury (Colonna & Butovsky, 2017; Lenz & Nelson, 2018). Under normal conditions they are ramified (have branches); however, microglia become activated under different injury states (Colonna & Butovsky, 2017). When an injury to the brain occurs, microglia become activated taking on a classical/neurotoxic activation state (M1) or an alternative/anti-inflammatory activation state (M2) (Sica & Mantovani, 2012). M1 microglia can be activated by interferon- gamma (IFN-γ) and toll like receptor (TLR) which leads to M1 microglia releasing neurotoxic factors such as tumor necrosis factor- alpha (TNF-α) and interleukin 6 (IL-6) (Figure 10) (Colonna & Butovsky, 2017). M2 microglia are activated by interleukin 4 ,13, and 10 (IL-4, IL-13, IL-10) as well as proliferator activated receptor gamma

(PPAR γ), in turn M2 microglia release anti-inflammatory factors such as IL-10 and transforming growth factor-beta (TGF-β) (Figure 10) (Colonna & Butovsky, 2017).

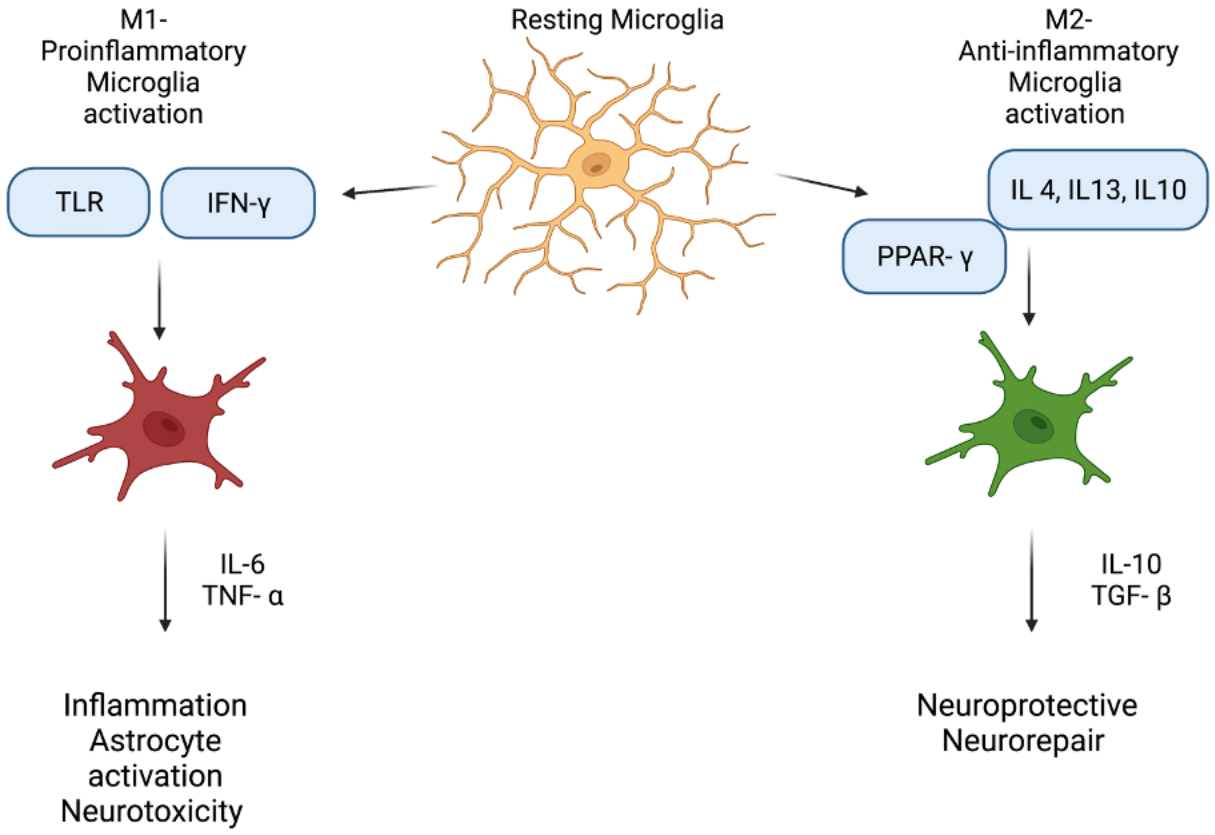


Figure 10: Activation of Microglia

This schematic depicts the different states of microglia activation and the associated factors and effects. TLR-toll-like receptor, IFN-γ- interferon- gamma, IL6- interleukin-6, TNFα- tumor necrosis factor- alpha, IL4, IL13, IL10- interleukin 4, 13, 10, PPAR- γ- proliferator activated receptor gamma, TGF-β- transforming growth factor-beta. Created with BioRender.com

Astrocytes are the most abundant non-neuronal glial cell, accounting for 20-40% of glial cells in the CNS (Westergard & Rothstein, 2020). Under normal conditions astrocytes play a plethora of roles including: regulating blood flow; maintaining homeostasis of ions, water and

pH; are involved in blood brain barrier formation and function; modulation of neuronal activity; and synapse homeostasis (Matias et al., 2019; Sofroniew & Vinters, 2010) (Figure 11).

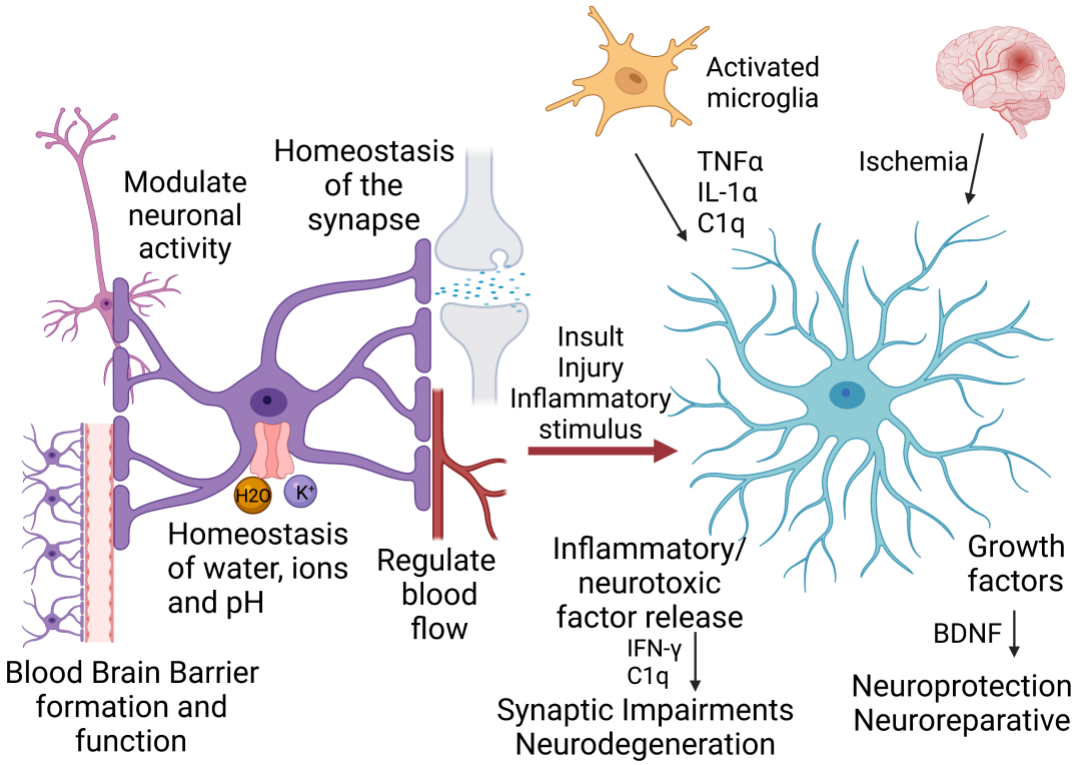


Figure 11: Overview of astrocyte function under normal conditions and following injury

Astrocytes play a number of important roles in normal function; however, upon injury they become reactive, changing their morphology and function as described elsewhere (K. Li et al., 2019; Matias et al., 2019; Sofroniew & Vinters, 2010). Created with BioRender.com

Astrocytes can be classified as protoplasmic which is typically found in gray matter with "fine" processes, while fibrous astrocytes are found in white matter with long "fibrous" processes as noted by Ramon and Cajal, (1909) in Sofroniew & Vinters (2010) and in Ramon & Cajal (1913). Astrocytes have a multitude of functions including the release of many important factors such as, thrombospondins (TSP), secreted protein acidic rich in cysteine (SPARC), SPARC-like1 (Hevin), transforming growth factor-beta (TGF-β), tumor necrosis factor-alpha

(TNF-α), and glypican 4 and 6 (Chung et al., 2015). which are involved in synaptogenesis, synaptic stabilization, and maturation (Christopherson et al., 2005; Perez-Catalan et al., 2021; M. L. Risher et al., 2015). The ability for astrocytes to release these factors following an insult to the brain shows the flexibility within the CNS to form new synapses; however, the formation of new synapses (synaptogenesis) following an insult can result in changes in synaptic plasticity (strengthening or weakening of synapses). Alterations to synaptogenesis and synaptic plasticity can disrupt vital homeostatic functions in the CNS. For example, under normal conditions glutamate and adenosine triphosphate (ATP) are important for synaptic function (Halassa et al., 2007). Glutamate is the most plentiful amino acid in the CNS and has excitatory effects on nerves, while ATP is a major energy source for functions such as intra-/intercellular signaling and amino acid activation (Neupane et al., 2019; Zhou & Danbolt, 2014). Glutamate clearance is regulated through high-affinity, sodium dependent excitatory amino-acid- transporters GLAST (EAAT1) and GLT-1 (EAAT2), glutamate transporters which are found on astrocytes particularly in the areas of the forebrain like the hippocampus, and cortex (Figure 12) (Anderson & Swanson, 2000; Danbolt, 2001). Additionally, it has been shown that EAAT2 or GLT-1 is responsible for 90-95% of glial glutamate clearance in the brain (Pregnolato et al., 2019; Yi & Hazell, 2006). Anderson & Swanson (2000) show astrocytes play an important role in glutamate uptake in normal conditions; however, uptake can be altered in disease states when transporter activity is inhibited by reduced ATP production. Ultimately, these functions give rise to the tripartite synapse where astrocytic endfeet are found ensheathing the pre- and post-synaptic terminals (Halassa et al., 2007). This is important as the astrocytic endfeet are vital for glutamate clearance from the extracellular space. When alcohol is introduced to the developing adolescent brain, it can disrupt glutamate clearance and astrocyte function (Adermark & Bowers, 2016).

Further, previous work has shown that alcohol can have an acute inhibitory effect on N-methyl-D aspartate (NMDA) glutamate receptors; however, upon receptor disinhibition, can lead to alterations in receptor function and increased receptor expression, increasing the potential for excitotoxicity and cell death (Figure 12) (Gonzales & Jaworski, 1997). EtOH also contributes to glutamate excitotoxicity (i.e. excessive glutamate release leading to neuronal hyperexcitability and cell death), a rat study using an intermittent 3-bottle choice paradigm with 2 bottles of H_2O and 1 bottle of 10% EtOH over 28 days by Blaker et al. (2019) showed that EtOH decreases GLAST expression but not GLT-1 24 hrs. after EtOH exposure. Further, they found increased basal glutamate levels pointing towards decreased glutamate uptake through astrocyte bound glutamate transporters in the striatum (Blaker et al., 2019). This increase in extracellular glutamate contributes to EtOH induced excitotoxicity following intermittent EtOH exposure, as well as in different stages of withdrawal (Figure 12) (Blaker et al., 2019; Gonzales & Jaworski, 1997). Interestingly, a study by Healey et al. (2021) shows male and female rats who were exposed to AIE between PND 30-46 then underwent a 24-day washout have increased glutamate transporter (GLT-1) expression in the dorsal hippocampus (DH) and increased GLAST expression in the DH of males; however, GLAST was decreased in the DH of females at PND70 (Figure 12). These findings by Healey et al. (2021) and Blaker et al. (2019) show alterations in glutamate transporter expression which can ultimately contribute to excitotoxicity. Further these results highlight potential compensatory mechanisms (increase in transporter expression) due to changes in proximity between the astrocyte and synapse. This change in glutamate clearance may contribute to altered excitability and long-term changes in synaptic function. Together, glia and neurons have well-regulated and coordinated interactions which ensure proper CNS function

and homeostasis. Disruption of one or more of these processes can lead loss of vital homeostatic functions.

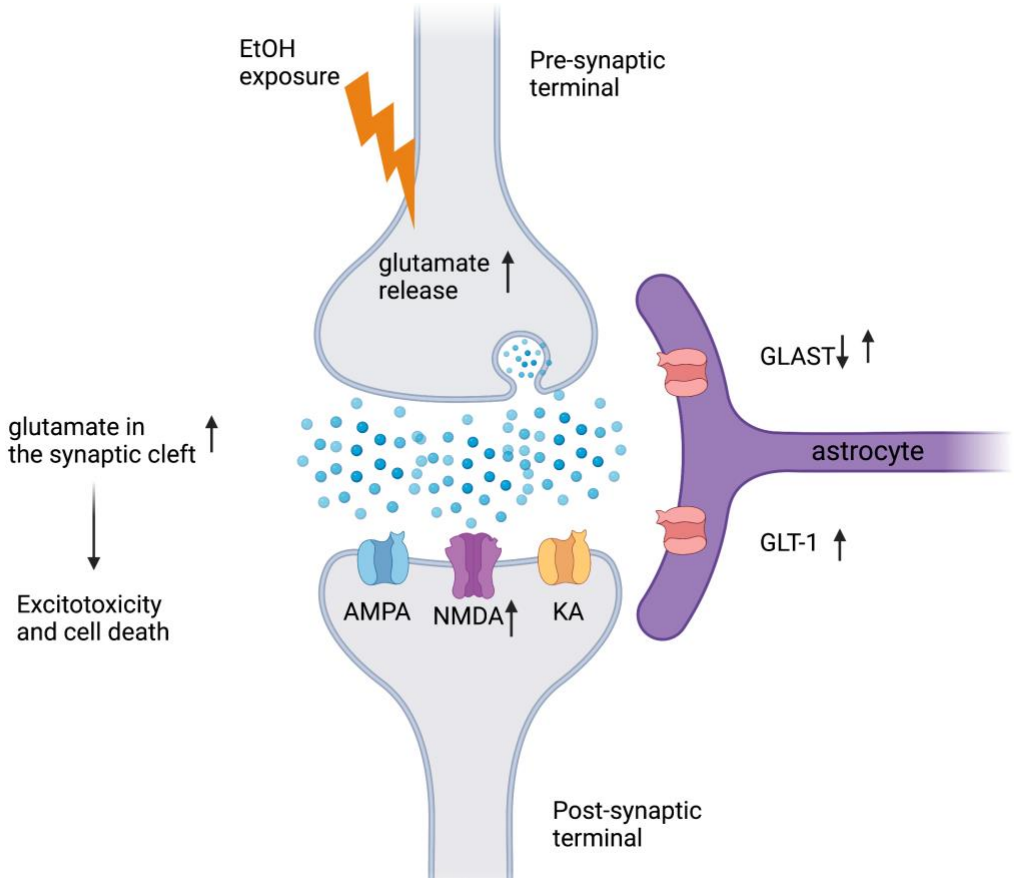


Figure 12: Glutamate and astrocyte function following EtOH exposure

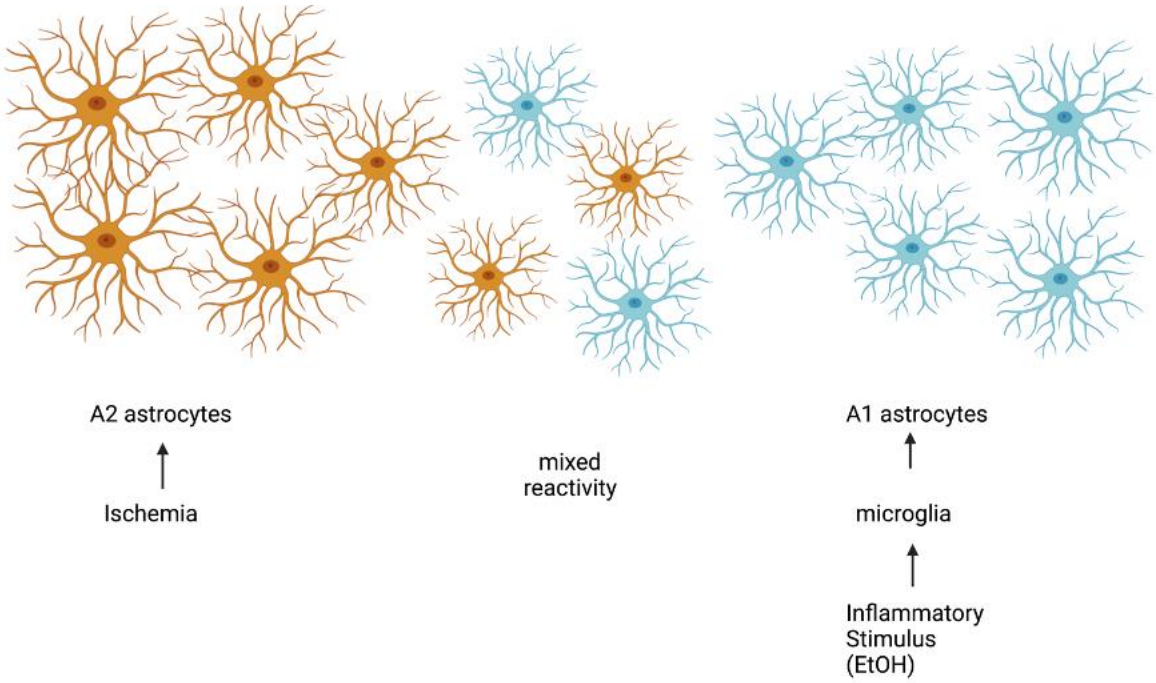
Following repeated EtOH exposure there is increased glutamate release and alteration in glutamate transporter function. This change in glutamate transporter and acute inhibition of NMDA and chronic increase in NMDA receptor expression and function can alter glutamate clearance which increases glutamate in the synaptic cleft, ultimately leading to excitotoxicity and cell death. Created with BioRender.com

Astrocyte Structure And Function Following Injury

Astrocyte structure and function is altered under injury, insult, or disease states. This change in structure and function is called reactive astrogliosis (Liddelow & Barres, 2017; Sofroniew & Vinters, 2010). Reactive astrocyte nomenclature is referred to as A1 which has shown to be proinflammatory, and A2 which has shown to be neuroprotective follows the nomenclature of microglia (M1/M2) (Liddelow & Barres, 2017). These states represent the two extremes of the astrocyte reactivity spectrum; however, there are other activation states of astrocytes which are yet to be fully elucidated (Liddelow & Barres, 2017; Liddelow et al., 2017). Further, Liddelow and Barres (2017) note that there can be a mixture and/or predominately A1 or A2 reactive astrocytes following injury; however, type of injury, severity and activation pathways involved will determine the type of reactivity (Figure 13A). Analysis of gene transcriptome data revealed the unique difference in A1 and A2 reactive astrocytes (Liddelow & Barres, 2017). A2 reactive astrocytes are induced by ischemia, with studies showing its signaling is through the signal transducer and activator of transcription 3 (STAT3) pathway, which in turn leads to the release of neurotrophic factors such brain derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF) ultimately leading to neuroprotection and repair (Figure 12B) (Baldwin & Eroglu, 2017; K. Li et al., 2019; Liddelow & Barres, 2017). An elegant study by Liddelow et al. (2017) found that A1 astrocyte reactivity is induced when microglia release tumor necrosis factor-alpha (TNF-α), complement component 1q (C1q), and interleukin-1alpha (IL-1α) which lead to inflammatory/neurotoxic/neurodegenerative effects. Activation of A1 astrocytes occurs through the Nuclear Factor-kappa B cell pathway (NF-κB) which leads to the release of neurotoxic/proinflammatory factors like interferon- gamma (IFN-γ) and C1q leading to neurodegeneration, and neurotoxicity (Figure 13B) (Baldwin & Eroglu, 2017; K. Li et

al., 2019). Liddelow et al. (2017) revealed that A1 astrocyte reactivity cannot be induced by TNF-α, C1q, IL-1α, alone or by only two of the three factors, all three must be released in order to induce A1 reactive astrocytes. However, in order to fully understand how these three factors contribute to activation of neurotoxic/proinflammatory astrocytes, it is imperative to understand the individual functions and pathways of TNF-α, C1q, and IL-1α .

13A


A2 astrocytes
Ischemia
mixed
reactivity
A1 astrocytes
microglia
Inflammatory
Stimulus
(EtOH)

13B

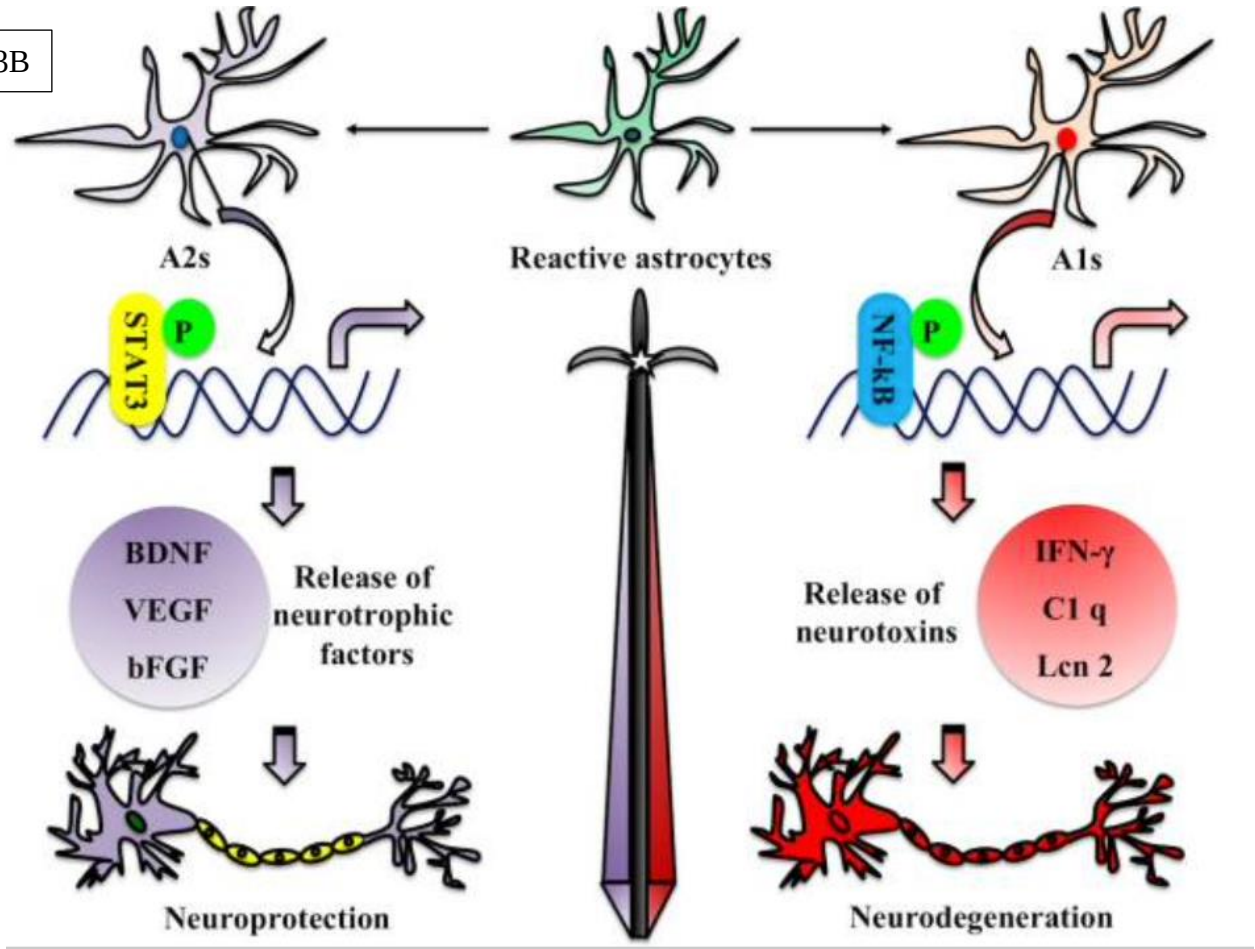

A2s
Reactive astrocytes
A1s
STAT3
P
NF-kB
P
BDNF
VEGF
bFGF
Release of
neurotrophic
factors
Release of
neurotoxins
IFN-γ
C1 q
Lcn 2
Neuroprotection
Neurodegeneration

Figure 13: States of astrocyte reactivity

A-There are different states of astrocyte reactivity including an intermediate mixed state. The most studied states are A1 and A2 reactivity. Created with BioRender.com B- A detailed schematic from Li et al. (2019) of how A1 or A2 astrocytes are activated and the associated factors released following activation.

The question/s that come about are: what are the functions of these three individual factors and why all three are necessary to induce A1 astrocyte reactivity? TNF-α, is an inflammatory cytokine that can lead to the activation of multiple signaling pathways (Idriss & Naismith, 2000). It can be expressed in soluble or transmembrane form which act through its p55 (TNFR1) and p75 (TNFR2) receptors (Idriss & Naismith, 2000; Taoufik et al., 2011). TNF-α plays a number of roles including being involved in sleep regulation, as administration of TNF-α increases sleep (Idriss & Naismith, 2000). Further, the TNF superfamily can mediate apoptosis, differentiation and proliferation through binding to one of its receptors (Aggarwal, 2003). For example, Aggarwal (2003) shows TNF binding to TNFR2 activates TNFR-associated factor 2 (TRAF2) which leads to downstream activation of the p38 mitogen-activated protein kinase (p38MAPK) and NF- κB through MAPK kinase 3 (MKK3) and inhibitor of NF- κB kinase (IKK) (Figure 14). Additionally, TNF binding to TNFR1 activates TNFR-associated death domain protein (TRADD) which leads to downstream activation of caspases through Fas associated death domain (FADD) (Figure 14) (Aggarwal, 2003). Each of these pathways respectively can lead to apoptosis and inflammation. However, it is important to note that these are not the only pathways that members of the TNF superfamily can activate, for an in-depth review see Aggarwal (2003). TNF has also been shown be neuroprotective, in a mouse model, Taoufik et al. (2011) found transmembrane TNF and inhibition of soluble TNF neuroprotective

against encephalomyelitis; however, it is dependent on neuron and astrocyte contact. TNF-α can also act through NF- κB and its p55 or TNFR1a receptor to trigger cell death (Hayden & Ghosh, 2014; Idriss & Naismith, 2000). These findings by Hayden & Ghosh (2014) and Idriss & Naismith (2000) taken with those of Taoufik et al. (2011) shows the mechanisms of TNF-α mechanisms can be a double-edged sword. However, TNF-α has classically been known to be proinflammatory and neurotoxic. A previous study using microglia conditioned medium treated with TNF-α shows activated microglia as well as markers for neuronal death, further they show TNF-α is a key promoter of excitoneurotoxicity via glutamate release from microglia (Takeuchi et al., 2006). TNF-α plays a multitude of functions some of which is mediated through astrocytes; however, by itself, TNF-α is not sufficient to induce A1 astrocyte reactivity (Liddelow et al., 2017). Similarly, interleukin-1α (IL-1α) comes from the interleukin-1 family of cytokines which are known to be involved in inflammatory pathologies (Malik & Kanneganti, 2018). Interestingly, intracellular IL-1α circulation within the body is commonly a sign of disease, while membrane IL-1α is found on several cells and tissue and is biologically active (Dinarello, 1996). Upon insult/injury IL-1α acts through its receptor IL-1R activating an inflammatory response through NF- κB and mitogen activated protein kinase (MAPK) pathways leading to the release of COX2 and TNF (Malik & Kanneganti, 2018). Further, binding of IL-1α to IL-1R leads to signal transduction through interleukin 1 receptor-associated kinase 1 (IRAK) and TNF receptor-associated factor 6 (TRAF6) which leads to activation of NF- κB also leading to inflammation (Figure 14) (Kaneko et al., 2019). Additionally, an elegant review by Achur et al. (2010) shows that cytokines TNF-α and IL-1α, as well as others have been implicated as serum, plasma and brain biomarkers of alcohol use and related tissue damage. This is important as researchers and clinicians continue to search for early signals of alcohol use and abuse on the

body, it may also point to a method of early detection for adolescents with potential alcohol related brain and tissue damage. However, just as TNF-α or IL-1α alone is not sufficient to induce A1 astrocyte reactivity, nor is TNF-α and IL-1α together (Liddelow et al., 2017). Complement component 1q (C1q) which is produced by macrophages is one of the many complement factors involved in the complement cascade (Reid, 2018). The complement cascade is a pathway involved in the innate immune system, and involves three different pathways: the classical (antigen/antibody) pathway, the alternative (hydrolysis) pathway, and the lectin (pathogen-associated molecular pattern molecules/ PAMP) pathway (Dunkelberger & Song, 2010). Activation of one or more of these pathways can lead to inflammation, tagging cells for phagocytosis, and cell death (Dunkelberger & Song, 2010). However, this review will only cover the classical and alternative pathways, for an in-depth review of all three pathways see Dunkelberger & Song (2010). C1q activates the classical complement pathway though the binding of the globular heads of C1q to antigen immunoglobulins (Celik et al., 2001; Dunkelberger & Song, 2010). While the alternative pathway is activated by hydrolysis of complement 3 (C3), the activation of these pathways will lead to either inflammatory, cell death or tagging mechanisms (Celik et al., 2001; Dunkelberger & Song, 2010). In particular, binding of the globular heads of C1q to immunoglobulins cleaves C2 and C4 whose products form C3 convertase leading to inflammation (Figure 14) (Dunkelberger & Song, 2010). Ultimately, the products of C3 convertase from the classical and lectin pathway, and C5 convertase from the lectin and alternative pathway can lead to cell death, inflammation or opsin tagging, for example cleaved C3a and C5a will lead to inflammation (Dunkelberger & Song, 2010). As noted with the previous two cytokines, C1q alone is not sufficient to induce A1 astrocyte reactivity (Liddelow et al., 2017). Each of these three factors (complement component 1q, interleukin-1α, and tumor

necrosis factor -α) plays a role in the immune response following insult, injury, or other inflammatory stimulus like alcohol and can ultimately lead to inflammation (Figure 14). Liddelow et al. (2017) thoroughly shows microglial release of all three factors (TNF-α, IL-1α, and C1q) are required for activation of A1 reactive astrocytes, with this information he developed a triple knockout mouse (TNF-$\alpha^{-/-}$, IL-1$\alpha^{-/-}$, C1q $^{-/-}$).

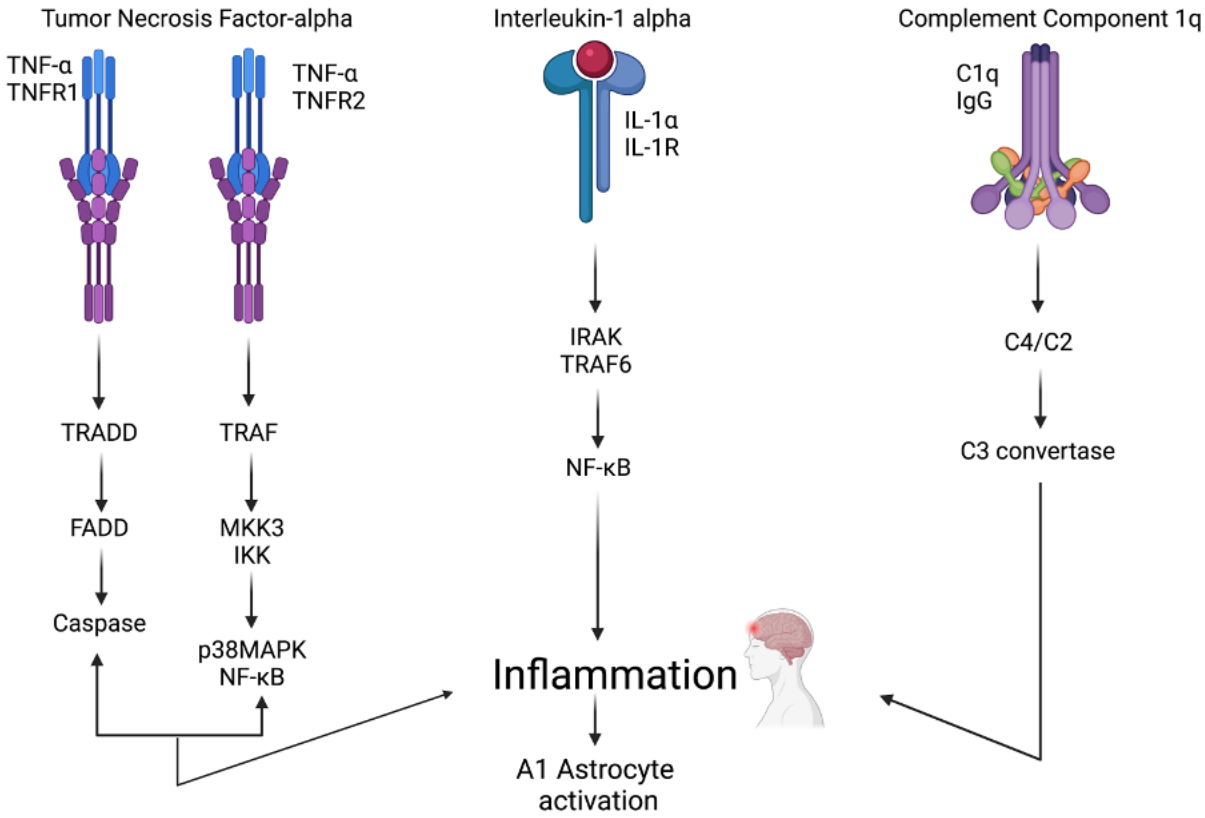


Figure 14: Pathways involved in TNF-α, IL-1α, and C1q induced inflammation and A1 astrocyte activation

These three factors bind to their respective receptors initiating several pathways which lead to increased inflammation in the brain. TRAF2-TNF binding to TNFR2 activates TNFR-associated factor 2, MKK3-MAPK kinase 3, IKK-inhibitor of NF- κB kinase, p38MAPK- mitogen-activated protein kinase, TRADD-TNFR-associated death domain protein, FADD-Fas associated

death domain, IRAK-Interleukin 1 receptor-associated kinase 1 and TRAF6-TNF receptor-associated factor 6, NF-κB-Nuclear Factor-kappa B cell pathway, C1q-Complement component 1q, IgG- immunoglobulin, C4/2-complement component 4/2, C3- complement component 3. Created with BioRender.com

Development Of An Astrocyte Specific And Behavioral Mouse Model Of Adolescent Alcohol Exposure

To date there are few astrocyte specific investigations of adolescent binge ethanol exposure using a mouse model. Much of the previously mentioned work has been done in rats; however, a mouse model offers the ability to use genetic modification for these types of studies. Our model uses the triple knockout (3KO) mouse developed by Shane Liddelow (New York University, NYU) and the AIE exposure paradigm with slight modifications as described by Risher et al. (2015) from PND 30 to 46. This model allows us to investigate the role of microglial released TNF-α, IL-1α, and C1q in astrocyte activation and reactivity following adolescent alcohol exposure. This is particularly important as previous work has shown that adolescent ethanol exposure leads to partial activation microglia that persists into adulthood (McClain et al., 2011). In short, the authors found that some but not all microglia transitioned to an activation state as indicated by significant changes in morphology (McClain et al., 2011).

It is well documented that alcohol leads to altered anxiety-like behavior, altered locomotor activity, alters sensitivity to reward as well as motivation for a reward (Kliethermes, 2005; Waeiss et al., 2019). Therefore, we incorporated tests that evaluate each of these noted changes. In order to assess the effects of AIE on locomotor and anxiety-like behavior we first used the open field assay, which is a highly used and well-studied test for locomotor and anxiety-like behavior (La-Vu et al., 2020; Seibenhener & Wooten, 2015). The open field box is typically

a clear plexiglass or white box with high walls where the animal can explore with no obstructions or objects in the box that is brightly illuminated (Figure 15). Measures such as distance traveled, average speed, grooming, freezing and time in thigmotaxis (time in the zone closest to the walls of the box) are some of many factors that can be assessed during this test (La-Vu et al., 2020). Total distance traveled and average speed offer insight into the locomotor behavior of each animal. While freezing and time in thigmotaxis offer good insight to anxiety-like behavior, since animals that are anxious spend more time in the thigmotaxis zone than in the center of the apparatus. To further assess the interactions between AIE, astrocytes and anxiety-like behavior we conducted the light dark assay. This assay consists of a dark (black) chamber with little light, and a clear or white chamber which is brightly illuminated with a small cut for the animal to travel through to switch chambers (Figure 15). Factors such as time in the light as well as time in the dark are common assessments for this model. Time in the light is commonly associated with decreased anxiety, while time in the dark is commonly associated with increased anxiety. In the case of increased anxiety the animal is more apprehensive about exploring the environment that is brightly lit and spends more time in the dark zone (La-Vu et al., 2020). The final assessment is conditioned place preference, which is commonly used Pavlovian conditioning to evaluate the relationship between contextual cues associated with reward-motivated behavior (McKendrick & Graziane, 2020). It involves the association of an environment (chamber) with a drug as well as a control chamber that is not associated with a drug. Conditioned place preference (CPP) is achieved when an animal spends more time in the drug paired chamber (Figure 15) (Prus et al., 2009). When a drug is rewarding animals should positively associate the drug with the paired chamber during conditioning phases, therefore during final testing animals will prefer the chamber associated with the rewarding drug. When a

drug is aversive the animal will negatively associate the drug with the drug paired chamber during conditioning, thus on the final testing day animals will prefer the non-drug paired chamber over the drug paired chamber (Prus et al., 2009). It is important to note that nicotine has both rewarding (low-moderate doses (0.2-0.6 mg/kg)) and aversive (high effects (0.8 mg/kg or above)) however these effects depend on dose, sex, rodent species and strain (Matta et al., 2007; O'Dell & Khroyan, 2009). Additionally, this CPP/CPA assessment engages the hippocampus which is the brains hub for learning and memory formation (Nam et al., 2019). It is important to note that other brain regions such as the amygdala, prefrontal cortex and cerebellum are key areas involved in learning and memory (Thompson & Kim, 1996). Further, Nam et al., (2019) found astrocytic μ −opioid receptors (MOR) in the hippocampus are required for CPP and reward based contextual memory formation. Interestingly, MOR's are not the only receptors that are expressed on astrocytes that can play a role in CPP, previous work has shown that astrocytes also express alpha 7 ($\alpha 7$) nicotinic acetylcholine receptors (nAChRs), showing astrocytes express a diverse collection of receptors where addictive substances can bind to. These findings are important as it suggests a possible role for astrocytes in reward preference through CPP.

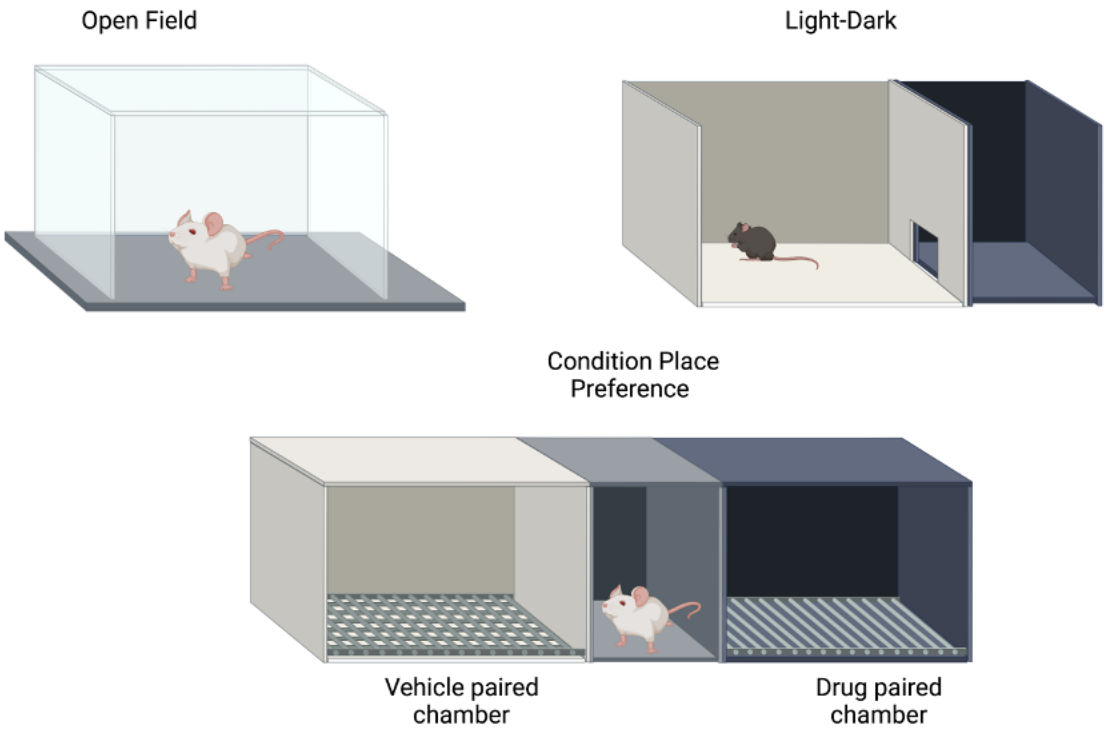


Figure 15: Behavioral Assays

This figure depicts the three different behavioral assays that are used to assess anxiety-like behavior, locomotor behavior and conditioned place preference. Created with BioRender.com

Additionally, this conditioning assessment allows us to investigate the relationship between TNF-α, IL-1α, and C1q induced activation of astrocytes and reward sensitivity. A recent review by Moriarity et al. (2020) shows the relationship between inflammation and reward sensitivity, as high and low reward sensitivity as well as reward drive (motivation to pursue a reward) may be associated with inflammatory related stress and the stressors involved in pursuing the reward. This is important as all three factors in our model are involved in inflammation, the removal of these three factors may drive increased preference for nicotine while decreasing inflammation and the negative association between reward, stress, and

inflammation. One potential overarching hypothesis is that decreasing A1-related inflammation can in turn decrease anxiety, decreases in anxiety leads to decreases in stress, decreases in stress can lead to decreased stress-induced inflammation and anxiety (Liu et al., 2017; Moriarity et al., 2020). This decrease in stress, anxiety and inflammation may in turn allow for positive associations with nicotine (increasing reward preference) (Figure 16) (Liu et al., 2017; Moriarity et al., 2020). Ultimately, this project provides a new and novel insight to the fields of alcohol, adolescent alcohol, reward, microglia, and astrocytes.

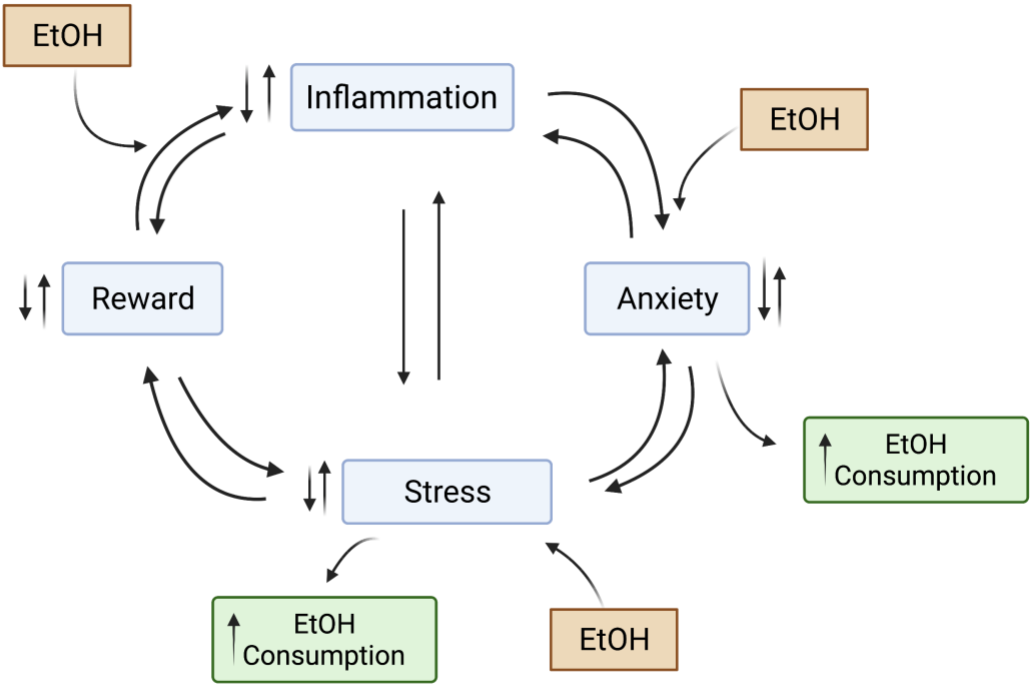


Figure 16: Circular and reciprocal interactions between inflammation, stress, anxiety and reward

Increases or decreases in inflammation can alter anxiety, stress, and reward motivated behavior. EtOH can increase inflammation and lead to anxiety and stress which in turn can lead to

increased EtOH consumption. In our 3KO model removal of three factors involved in the inflammatory response may decrease inflammation which in turn decreases anxiety and stress leading to positive association with a rewarding stimulus. For a more detailed explanation please see the discussion in chapter 4. Created with BioRender.com

Conclusion

Alcohol use and abuse is prevalent among adolescents and can lead to effects that last into adulthood. Human studies and animal studies provide critical information as to the acute and long-term cellular, molecular, and behavioral effects of alcohol exposure. These studies provide crucial background for the adolescent alcohol studies. Further, studies by Risher and colleagues and Liddelow and colleagues provide the rationale and background for our study as these studies provide key information on astrocyte reactivity as well as AIE induced changes to astrocyte and microglia structure and function (Liddelow et al., 2017; McClain et al., 2011; M.-L. Risher et al., 2015; M. L. Risher et al., 2015). Though many advances have been made in studying alcohol and in intervention measures, there is still much to learn in relation to the underlying mechanisms driving alcohol related changes in the brain.

CHAPTER 4

THE INTERSECTION OF ALCOHOL, GLIA AND BEHAVIOR: CAN ASTROCYTES INFLUENCE ANXIETY-LIKE AND REWARD-MOTIVATED BEHAVIOR FOLLOWING ADOLESCENT ETHANOL EXPOSURE?

A manuscript in preparation

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Abstract

Early onset binge drinking increases the likelihood of developing an alcohol use disorder later in life. However, the underlying cellular mechanisms that drive these changes are not well understood. Recent work has implicated neuroimmune glial processes in the development of alcohol-induced changes in brain function; however, there remain significant gaps in knowledge regarding the relationship between neuroimmune activation and the development of sensitivity to addictive substances. Previous work in our laboratory and others have demonstrated that microglia and astrocytes display stereotypical reactive phenotypes in response to repeated ethanol exposure. Of particular interest are interleukin-1 alpha (IL-1α), tumor necrosis factor-alpha (TNF-α), and complement component 1q (C1q); proteins involved in the induction of detrimental microglial-induced astrocyte reactivity. Here we investigated the role of this pathway in reward-sensitivity and anxiety-like behavior following repeated adolescent EtOH exposure. At postnatal day (PND) 30 male and female C57BL/6J mice and triple knockout mice (3KO, ~ TNF-α, C1q, IL-1α) received ethanol (EtOH, 5.0 g/kg i.g.) or water intermittently over 16 days. Following a 7-day washout period, mice underwent a battery of behavioral tests consisting of open field, light-dark box, and conditioned place preference (CPP) to test locomotor activity, anxiety, and reward sensitivity for nicotine, respectively. Our results show unique genotype specific differences particularly in our females, showing that the 3KO female mice demonstrate decreased anxiety and increased preference for reward compared C57BL/6J females. Interestingly, our male data shows mixed or no significant differences, highlighting sex specific sensitivities to nicotine. Additionally, immunohistochemical staining and analysis reveals sex and genotype specific differences in microglia, astrocytes and in complement component expression. These data show unique genotype differences in the sex-dependent regulation of

anxiety-like behavior, reward-sensitivity, and neuroinflammatory response following binge EtOH exposure. Further, this study sheds light on the potential roles and interactions between glial cells, sex, and behavior in response to addictive substances.

Key words: glia, astrocytes, adolescent, ethanol, mice, neuroimmune

Introduction:

Adolescence is a critical developmental stage hallmarked by risky-decision making, sensation seeking, and cognitive development (Spear, 2000). This time of developmental transition is correlated with maturation in brain regions important for learning, memory, decision-making, and planning (Larsen & Luna, 2018). It is also a period in which alcohol use in adolescence remains prevalent, with over 25% of 15-19 year-olds reporting to have consumed alcohol within the last month (World Health Organzation, 2018). Binge drinking, which is defined as 5 or more drinks for males and 4 or more drinks for females in a two-hour period, increases across adolescence, particularly from 8^{th} grade (13-14 years-old) to 12^{th} grade (17-18 years-old) (Johnston et al., 2016). Further, evidence shows that both adolescent humans and rodents are less sensitive to the motor impairing effects of acute alcohol but are more sensitive to the rewarding effects of acute alcohol (Ramirez & Spear, 2010; Spear, 2014). Additional human studies show adolescents are more affected by alcohol related deficits in learning and neural development than adults (Lees et al., 2020). While other human studies reveal a significant correlation between learning and memory deficits and level of alcohol use in adolescence (Hanson et al., 2011).

Rodent studies have shown a wide variety of EtOH-induced behavioral effects. An adult alcohol/ethanol (EtOH) study which exposed 8-9 week old C57BL/6J mice to a 20% EtOH two-bottle choice paradigm for 7 weeks found no changes in anxiety like behavior in both males and

females; however, they noted that the females showed less anxiety than males (Bloch et al., 2020). While a prenatal alcohol exposure study, which introduced 2.8 g/kg of 23.7% EtOH (2x) at gestational day 8 leads to alterations in behavior in adulthood (Fish et al., 2018). Interestingly, a recent adolescent intermittent ethanol (AIE) study demonstrated that female mice showed increased anxiety-like behavior following short- (anxiety tests between PND 79-91) and long-term (anxiety tests between PND 100-102) abstinence periods and voluntary EtOH consumption while males only show anxiety at the long-term timepoint (Maldonado-Devincci et al., 2021). These studies reveal that timing of EtOH exposure is a critical factor and that repeated adolescent EtOH exposure results in acute and long-term changes in EtOH preference in adolescence and behavioral changes that persist into adulthood.

Many AIE or chronic intermittent ethanol (CIE) studies have also been conducted in rats. Several rat studies have shown that AIE / CIE exposure leads to long-term molecular and cellular changes in the brain and effects EtOH sensitivity, consumption and behavior which persist into adulthood (Gass et al., 2014; Risher et al., 2013; M.-L. Risher et al., 2015; M. L. Risher et al., 2015; Varlinskaya et al., 2020). A study by Gass et al. (2014) shows that adolescent rats exposed to intermittent EtOH via vapor chambers between PND 28-42 leads to increased ethanol self-administration in adulthood, and increases the amount of time spent in the open arms of the elevated plus maze, showing AIE can reduce anxiety in this model. In addition to the various behavioral and cognitive effects of AIE, recent studies have shown that AIE cause alterations to specific non-neuronal cells. A study by Risher et al. (2015) which exposed rats to AIE between PND 30-46, found that AIE leads to long-term morphological changes in astrocytes. Further, this study found increased synapse formation (synaptogenesis) as well as upregulation of thrombospondin 2 and 4 which are astrocyte secreted proteins that are critically involved in

developmental and adult synaptogenesis (Eroglu et al., 2009; M. L. Risher et al., 2015). This upregulation of synaptogenic factors appears to drive synaptic remodeling following AIE (M. L. Risher et al., 2015) as well as changes in synaptic function (Ziv & Brenner, 2018). Further, these newly formed synapses can mature/stabilize and can integrate into the existing circuitry, increase receptor expression, and drive the motivation to seek the drug of abuse (Russo et al., 2010).

Recent animal studies have implicated astrocytes as well as microglia in ethanol induced neuroinflammation (Guerri & Pascual, 2019; Pascual et al., 2018; Rubio-Araiz et al., 2017). Microglia and astrocytes are both intricately involved in neuroinflammatory processes in the brain. Microglia respond to injury, insult, and inflammation by becoming activated (ramified) and releasing multiple signaling factors that in many cases can be detrimental to neuronal and synaptic health and survival (Lenz & Nelson, 2018). Astrocytes, in addition to being important for the regulation of cerebral blood flow; balance of ions, fluid, and pH; synaptic formation and function, and blood brain barrier formation and function, also play a critical role in neuroinflammation through reciprocal signaling with microglia and neurons. Moreover, similar to microglia, astrocytes are activated following injury, insult, and inflammation (Sofroniew & Vinters, 2010). However, the characteristics of these activation pathways are highly complex and depending on the type of injury or inflammatory stimulus can result in a spectrum of microglial and astrocyte responses. In the past, the extreme ends of the activation spectrum have been described as reactive microglia (M1 and M2) and reactive astrocytes (A1 and A2) (Liddelow & Barres, 2017; Miguel-Hidalgo, 2018) with the latter being characterized by the unique changes in astrocyte function, the secretion of specific signaling factors, and morphometric changes. Interestingly, A2 reactive subtypes are deemed more neuroprotective following an insult and is thought to promote recovery of lost neuronal circuitry (Liauw et al., 2008; Liddelow & Barres,

2017). Whereas A1 reactive astrocytes are activated by factors released from microglia [interleukin-1 alpha (IL-1α), tumor necrosis factor-alpha (TNF-α), and complement component-1q (C1q)] resulting in synaptic impairment and neurodegeneration (Liddelow & Barres, 2017; Liddelow et al., 2017).

It has been demonstrated in both rodent and post mortem human studies that EtOH can induce the upregulation of interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor- alpha (TNF-α) (Fernandez-Lizarbe et al., 2009; Guerri & Pascual, 2019; Vallés et al., 2004), factors critically involved in the neuroinflammatory processes previously discussed. A study that exposed rats to a diet containing 5% EtOH for 5 months as well as using astrocyte cell cultures found increased IL-1 expression, caspase-3 expression, increased extracellular regulated-kinase (ERK1/2), p38 and Jun N-terminal kinase (JNK), as well as increased DNA binding of nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1) in both astrocytes from the cerebral cortex of EtOH fed rats and astrocyte cultures (Vallés et al., 2004). This study showed that EtOH exposure increases multiple factors involved in cell death and the inflammatory pathways. Further, AIE studies show adult rats exposed to AIE increases high-mobility group box 1 (HMGB1) through toll-like receptors (TLRs) and receptor for advanced glycation end products (RAGE) which results in downstream activation of AP-1 and NF-κB (Crews et al., 2016; Vetreno & Crews, 2012). This activation of AP-1 and NF-κB leads to increase inflammation, leading to activation of microglia and astrocytes (Crews et al., 2016). These data combined with previous studies demonstrating EtOH-induced astrocyte activation (Risher et al., 2015), suggest that microglia and astrocytes are intimately involved in neuroimmune responses to repeated AIE. However, microglial release of IL-1α, TNF-α, C1q which drive neurodegeneration-associated astrocyte activation have not been investigated in the context of adolescent EtOH exposure.

The role of neuroimmune activation in neuronal and synaptic remodeling begs the question of whether AIE induced-neuroimmune activation contributes to neuronal remodeling that predisposes rodents and humans to the increased the likelihood of developing substance use disorders in adulthood (Jordan & Andersen, 2017; Salmanzadeh et al., 2020). Of particular interest is future nicotine use. Recent work has shown that female rats exposed to AIE between PND 30-60 have increased the likelihood of nicotine use in adulthood (Waeiss et al., 2019), showing a direct link between alcohol consumption and future substance use. Though previous studies have shown that AIE has a plethora of effects including changes to the brain circuitry and cellular function as well as changes in anxiety-like and reward motivated behavior, there remain significant gaps in knowledge with regard to the contributions/role of non-neuronal glial cells in behavior. To date there are few mouse models investigating the effects of AIE on microglial-astrocyte immune-signaling and subsequent changes in reward preference. Here we use a genetically modified mouse model to investigate the effects of AIE on microglial-astrocyte signaling and the involvement of the IL-1α, TNF-α, and C1q proinflammatory pathways. Using a triple knockout mouse (3KO, TNF-$\alpha^{-/-}$, IL-1$\alpha^{-/-}$, C1q$^{-/-}$) we investigate the effects of AIE on astrocyte morphology, anxiety-like behavior, development of preference for nicotine, and glia-related protein expression. We hypothesize that loss of IL-1α, TNF-α, and C1q proinflammatory signaling alters astrocyte activation leading to decreased anxiety-like behavior while increasing reward motivated behavior. These data reveal a novel finding that the deletion of IL-1α, TNF-α, and C1q plays a significant role in reward and anxiety-like behavior despite observing limited effects of AIE on these behaviors.

Materials And Methods:

Mice

All experiments were conducted in accordance with guidelines for care and use of animals provided by the National Institutes of Health and were approved by the Huntington VA Medical Center and Marshall University IACUC. For behavioral assays a total of 121 male and female C57BL/6J (Hilltop, Scottdale, PA) and triple knockout mice (TNF-$\alpha^{-/-}$, IL-1$\alpha^{-/-}$, C1q$^{-/-}$) on a C57BL/6J background (provided by Shane Liddelow, New York University) were grouped housed (4-5 per cage) in a standard normal 12/12-hour light-dark cycle (light on at 6 am, lights off at 6 pm), and were maintained in a temperature and humidity-controlled room with *ad libitum* access to food and water. Three - five days prior to the start of dosing animals are handled by the experimenter. Using a modified protocol from Risher et al., (2015) starting at PND 30 mice are given 5.0g/kg EtOH i.g. or vehicle (H_2O) in a 2-days on, 1 day off, 2 days on, 2 days off paradigm for 16 days (PND 30-46). Animals were genotyped to confirm gene deletion (Jacquot et al., 2019). Following a 7-day washout period, animals underwent behavioral testing between PND 54-84 (Figure 17).

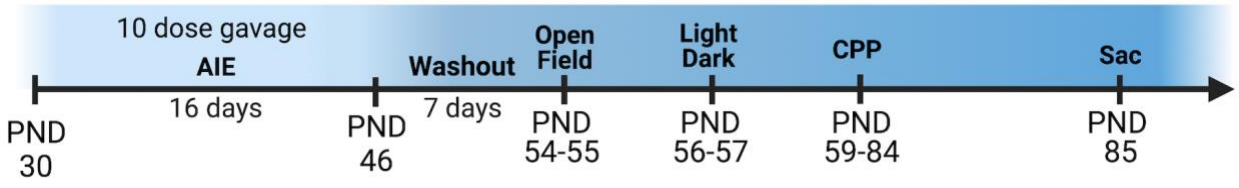


Figure 17: Experimental timeline

This timeline depicts the experimental timeline with post-natal day and associated assay

Behavioral assays

Open field

Male and female C57BL/6J and 3KO mice (121 animals) were moved to the testing room 30 minutes prior to testing for habituation. Animals were placed in the open field box, a clear 40 cm x 40cm walled box (Stoelting Co., Wood Dale, IL) which is illuminated between 25-30 lux. Animals were individually placed in the box and allowed to explore the apparatus for 10 mins. In between each animal the box was cleaned with 15% vinegar. Each animal was tracked using Any-maze video tracking software (Stoelting Co., Wood Dale, IL). Data was assessed for time spent in thigmotaxis (the zone closest the walls of the open field box) as an indicator of increased anxiety (Simon et al., 1994). Average speed (meters/second (m/s)) and total distance traveled (meters) were also assessed.

Light-dark assay

Male and female C57BL/6J and 3KO mice (120 animals) were moved to the testing room 30 minutes prior to testing for habituation. The light-dark box has two enclosures (40cm x 40cm) with one dark (black) walls and lid, and one clear with no lid that is illuminated between 40-45 lux (Stoelting Co., Wood Dale, IL). Animals were placed in the light side of the box and allowed to explore the apparatus for 10 minutes. Between each animal the box was cleaned with 15% vinegar. Videos were manually recorded then analyzed using Any-maze video tracking software (Stoelting Co., Wood Dale, IL). Data was assessed by time in the light, which examines the tendency to explore brightly illuminated open spaces and a novel environment as opposed to remaining in a dark “safe” area. (La-Vu et al., 2020)

Conditioned place preference assays

CPP assays were conducted using 116 male and female C57BL/6J and 3KO mice with a three-chamber place-preference apparatus (Harvard Apparatus, Halliston, MA) and were tracked using Any-maze video tracking software (Stoelting Co., Wood Dale, IL). Using a protocol as described by Cooper et al. (2020), an unbiased 10-day protocol using saline and 0.5 mg/kg of nicotine ((-)-Nicotine hydrogen tartrate, Sigma Aldrich 1463304, a gift from Brandon Henderson). Mice received 0.5 mg/kg nicotine (i.p.) on (days 2,4,6,8) or saline (days 3,5,7,9). Drug or saline was administered to mice immediately prior to being placed in white-gray striped (drug paired) chamber or in the white-black dotted chamber (saline paired). The assay took place in three stages: pre-test (day 1); injections (day 2-9), and post-test (day 10). During the pre-test, animals were placed in the center chamber with the guillotine doors removed and the mouse was given 20 minutes to explore the apparatus. Time spent in each chamber is recorded, any mouse that spends more than 65% of the time in a chamber were excluded (5 exclusions, 3 females (1-C57 EtOH-saline, 1-3KO H_2O-saline, 1- 3KO EtOH-nicotine), 2 males (1-C57 EtOH saline, 1-3KO EtOH saline) as described in (Cooper et al., 2020). On injection days, mice were placed in the drug paired chamber on even days and in the saline paired chamber on odd days for 20 minutes. During the post-test mice were placed in the center chamber, the guillotine doors were removed and mice were allowed to explore the apparatus for 20 minutes. Data was expressed as change in baseline preference between the post-test and pre-test.

Tissue preparation

Following all behavior tests mice were deeply anesthetized using isoflurane (Halocarbon, beech Island, SC). Mice were transcardial perfused for 5 minutes using 1X Phosphate Buffered Saline (PBS, Sigma Aldrich P5368) followed by 4% paraformaldehyde (PFA, Electron

Microscopy Sciences Cat. #19208). Brains were removed and placed in 4% PFA for 24 hours then washed with 1X PBS three times. Brains were then placed in a 30% sucrose solution for 1-3 days. Brains were frozen using a 2:1 30% sucrose solution and tissue freezing medium and stored at -80ºC. 40µm thick slices were sectioned (Leica CM1850, Wetzlar, Germany).

Immunohistochemistry

Three to six dorsal hippocampal slices per animal were blocked using 5% normal goat serum (NGS, Jackson ImmunoResearch, 005-000-121) or 5% normal donkey serum (NDS, Jackson ImmunoResearch, 017-000-121) in 0.1% or 0.2% phosphate buffered saline with Triton-X100 (PBST, Roche diagnostics- 11332481001) for 1 hour at room temperature. Primary antibodies were prepared in 5% NGS or NDS in 0.1% or 0.2% PBST. Primaries used: anti-complement 3 (C3, 1:200, Invitrogen, PA5-114921); anti-glial fibrillary acidic protein (GFAP, 1:500, abcam-ab53554); anti-ionized calcium-binding adaptor molecule 1 (Iba1, 1:500, Wako-019-19741). Confirmation staining using anti-tumor necrosis factor-alpha, (TNF-α ,1:100, proteintech-60291-1-Ig); anti-interleukin-1α, (IL-1α, 1:50, R&D systems, AF-400-NA), anti-complement component 1q (C1q, 1:1,000, Abcam-ab71089). Slices were incubated in primary antibody over night at 4ºC followed by secondary antibody incubation (1:200 or 1:500): goat anti-rabbit IgG, Alexa Fluor-594 (Invitrogen, A11012); goat anti-mouse IgG, Alexa Fluor-594 (Invitrogen, A11032); donkey anti-rabbit IgG, Alexa Flour-594 (Invitrogen, A21207), donkey anti-goat IgG, Alexa Fluor-594 (Invitrogen, A11058) or donkey anti-goat IgG, Alexa Fluor-647 (Invitrogen, A21447) for 2 hours at room temperature. Slices were washed in PBST, mounted onto microscope slides (Vector Laboratories, H-1200-10) and sealed with nail varnish. Slides were stored in the -20ºC freezer until imaging.

Imaging

Dorsal hippocampal slices were stained for GFAP and Iba-1 and were imaged using confocal microscope (Leica SP5 TCSII, Wetzlar, Germany) with z-stack (10 steps, 0.99 um step size, 1024 x 1024 image size) using a 20x objective (n =3/treatment). Dorsal hippocampal slices were stained for C3 (n=3-4/treatment), as well as TNF-α, IL-1α, and C1q for confirmation of knockout (n=1-2/treatment),and were imaged using the 63x objective (1024 x 1024 image size). Images were analyzed using FIJI-imageJ2 (NIH, Laboratory for Optical and Computational Instruments, University of Wisconsin Madison, WI) image processing software. Maximum projections of the optical sections were generated from the original z-stack. The region of interest was selected from an area within the Cornu Ammonis-1 (CA1) region of the dorsal hippocampus (DH) and the pixel intensity, mean, minimum, and maximum were measured. Astrocyte and microglia cell counts were done manually in the region of interest within the CA1 of the DH.

Statistical analysis

All results are presented as mean$\pm$SEM with all statistical analysis performed using GraphPad Prism (GraphPad Software Inc, San Diego, CA). Results are shown as one-way or two-way-ANOVA for statistical significance with Tukey *post hoc* for means comparison Statistical significance is an alpha level of 0.05.

Results

The effects of AIE and genotype on locomotor activity

Locomotor activity was assessed using the open field assay. Total distance traveled in meters (m) and average speed in meters per second (m/s) were assessed for both males and females. The ANOVA for total distance traveled for females reveals a significant treatment

effect (EtOH vs H_2O) ($F_{(1,57)}$=10.17, p=0.0023) and a genotype effect (3KO vs C57BL/6J) ($F_{(1,57)}$=35.23, p<0.0001). The post-hoc analysis reveals a significant decrease in distance traveled in the H_2O exposed 3KO females compared to the H_2O exposed C57BL/6J females (p=0.0001), in the EtOH exposed C57BL/6J females compared to the H_2O exposed C57BL/6J females(p=0.0458), in the EtOH exposed 3KO females compared to the H_2O exposed C57BL/6J (p<0.0001), and in the EtOH exposed 3KO females compared to the EtOH exposed C57BL/6J (p=0.0021) (Figure 18A). The ANOVA for total distance traveled for males reveals a genotype effect ($F_{(1,56)}$=5.660, p=0.0208) but no treatment effect ($F_{(1,56)}$=0.05447, p=0.8163) while post-hoc analysis revealed no further significant differences (Figure 18B). These data reveal that genotype appears to influence locomotor activity in female mice but not males, with little influence of prior EtOH exposure. In order to further assess changes in locomotor behavior we next assessed average speed (m/s).

When assessing average speed (m/s) in our females the ANOVA reveals a treatment ($F_{(1,57)}$=9.984, p=0.0025) and genotype effect ($F_{(1,57)}$=35.51, p<0.0001). Post-hoc analysis reveals a significant decrease in speed in the H_2O exposed 3KO females when compared to the H_2O exposed C57BL/6J females (p=0.0001), in the EtOH exposed C57BL/6J females compared to the H_2O exposed C57BL/6J females (p=0.0454), in the EtOH exposed 3KO females compared to the H_2O exposed C57BL/6J females (p<0.0001), and in the EtOH exposed 3KO females compared to the EtOH exposed C57BL/6J females (p=0.0021) (Figure 18C). The ANOVA for average speed for males reveals a genotype effect ($F_{(1,56)}$= 5.965, p=0.0178), while post-hoc analysis shows no further significant differences (Figure 18D). These data reveal a unique sex, genotype and treatment difference, it further reveals that our 3KO females appear to show hypo-locomotor activity compared to the C57BL/6J females irrespective of prior EtOH exposure. To

determine if hypo-locomotor activity was due to decreased anxiety we assessed time in thigmotaxis.

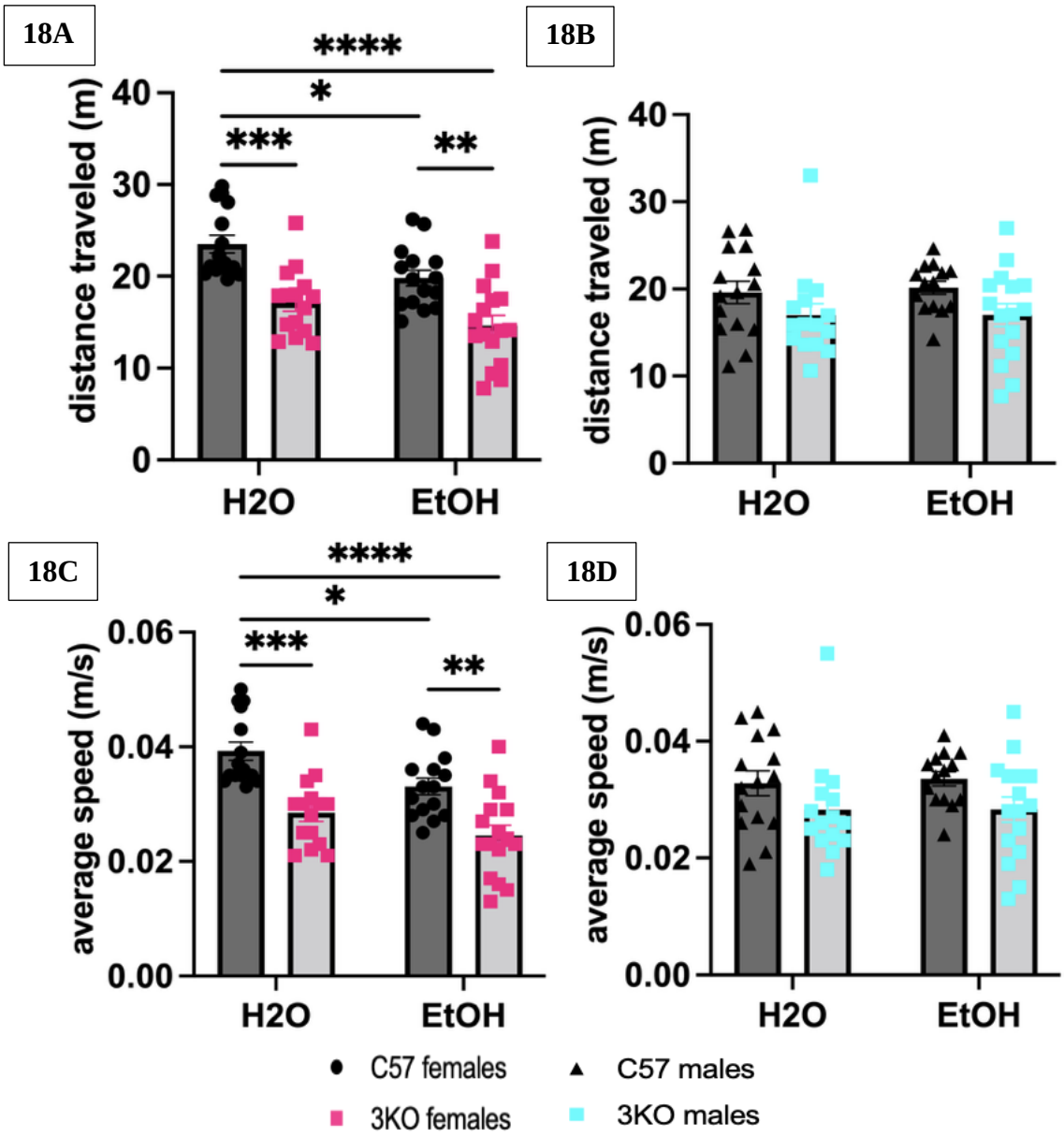


Figure 18: The effects of sex, genotype and treatment (AIE/H_2O) on locomotor behavior

A- Females: total distance traveled (m) for H_2O and EtOH exposed C57BL/6J (black dots, dark gray box fill), (H_2O+C57BL/6J and EtOH+C57BL/6J; n=15), and 3KO (pink squares, light gray

fill) (3KO+H_2O, 3KO+EtOH; n=15-16). B- Males: total distance traveled (m) for H_2O and EtOH exposed C57BL/6J (black triangle, dark gray fill) (C57BL/6J+H_2O, C57BL/6J+EtOH; n=14-15) and 3KO (light blue squares, light gray fill) (3KO+H_2O, 3KO+EtOH; n=15-16). C- Females: average speed (m/s) for H_2O and EtOH exposed C57BL/6J ((H_2O+C57BL/6J and EtOH+C57BL/6J; n=15), and 3KO (3KO+H_2O, 3KO+EtOH; n=15-16). D- Males: average speed (m/s) for H_2O and EtOH exposed C57BL/6J (C57BL/6J+H_2O, C57BL/6J+EtOH; n=14-15) and 3KO (3KO+H_2O, 3KO+EtOH; n=15-16). All data are shown as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.0021$, ***$p \leq 0.0002$, ****$p < 0.0001$

The effects of AIE and genotype on anxiety-like behavior: time in thigmotaxis

Time in thigmotaxis or time in the zone near the walls/periphery of the box and can be used as an indicator of anxiety-like behavior (La-Vu et al., 2020; Simon et al., 1994). The ANOVA for females reveals a genotype effect ($F_{(1,57)}=12.43$, p=0.0008) but no treatment effect ($F_{(1,57)}=0.4691$, p=0.4962). Post-hoc analysis reveals a significant decrease in time spent in thigmotaxis in the H_2O exposed 3KO females compared to the EtOH exposed C57BL/6J females (p=0.0229), and in the EtOH exposed 3KO females compared to the EtOH exposed C57BL/6J females (p=0.0260) (Figure 19A). The ANOVA for males revealed a treatment effect ($F_{(1,56)}=4.396$, p=0.0406), but no genotype effect ($F_{(1,56)}= 0.6282$, p=0.4314) while post-hoc analysis revealed no further significant differences (Figure 19B). Here our data reveal genotype and treatment effects, and shows that the 3KO females spend less time in thigmotaxis compared to the C57BL/6J female mice, pointing towards decreased anxiety in the 3KO females. Locomotion and anxiety have a complicated relationship as locomotor depression and hyper-locomotion can both be attributed to anxiety-like as well as withdrawal related behavior, making it important to conduct multiple locomotor/anxiety behavioral tests (Archer, 1973; Sestakova et

al., 2013). In order to further investigate the relationship between locomotor activity and anxiety, we assessed time in the light of the light-dark box.

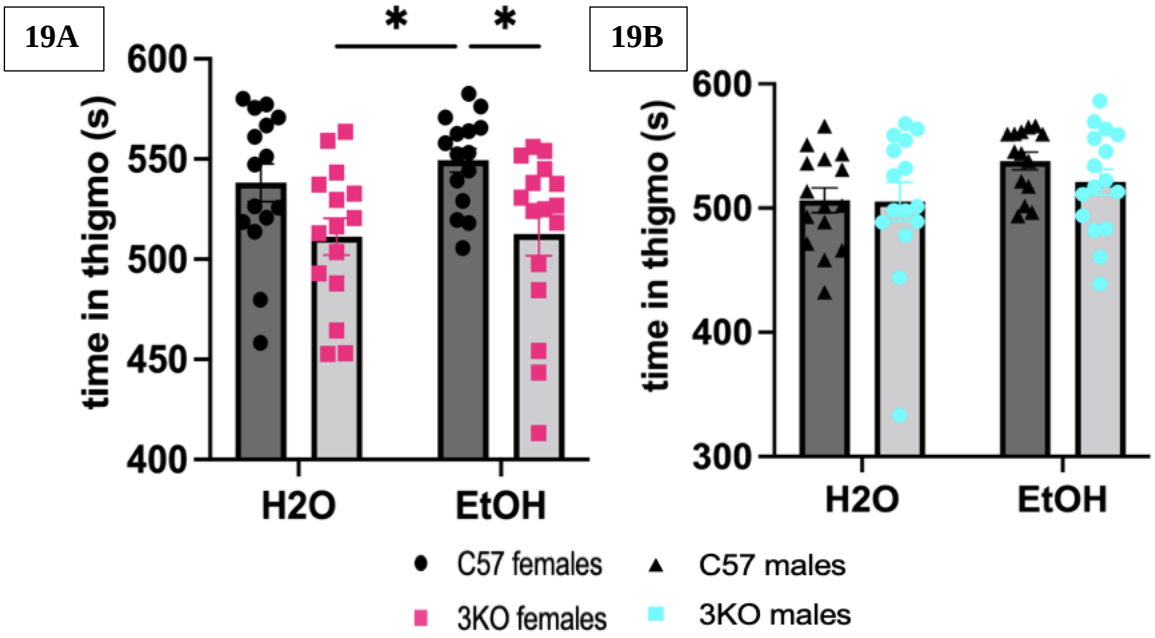


Figure 19: Determining anxiety like behavior: time in thigmotaxis

Time in thigmo (thigmotaxis) (s) which is near the walls of the open field box. A- Females: time in thigmotaxsis (s) for H_2O and EtOH exposed C57BL/6J (H_2O+C57BL/6J and EtOH+C57BL/6J; n=15), and 3KO (3KO+H_2O, 3KO+EtOH; n=15-16). B- Males: time in thigmotaxis (s) for H_2O and EtOH exposed C57BL/6J (C57BL/6J+H_2O, C57BL/6J+EtOH; n=14-15) and 3KO (3KO+H_2O, 3KO+EtOH; n=15-16). All data are shown as mean ± SEM. *$p \leq 0.05$

The effects of genotype, sex, and AIE on anxiety-like behavior

In order to investigate the relationship between hypo-locomotor activity and anxiety we assessed time in the light of the light-dark box. Light-dark is a simple assay which tests for anxiety-like behavior by avoidance of brightly illuminated open spaces and the preference of

dark spaces (La-Vu et al., 2020). For the females the ANOVA shows a genotype effect ($F_{(1,57)}$=10.85, p=0.0017) but no treatment effect ($F_{(1,57)}$=0.6960, p=0.4076). Post-hoc analysis reveals a significant increase in time spent in the light in the H_2O exposed 3KO females compared to the H_2O exposed C57BL/6J females (p=0.0132), and in the H_2O exposed 3KO females compared to the EtOH exposed C57BL/6J females (p=0.0267) (Figure 20A). The male data reveals no significant differences for the ANOVA, genotype effect ($F_{(1,55)}$ = 0.8237, p= 0.3681), treatment effect ($F_{(1,55)}$ =0.8674, p=0.3557), and post-hoc analysis revealing no further significant differences (Figure 20B). Overall, our open field and light-dark data demonstrate that 3KO females have decreased anxiety-like behavior compared to the C57BL/6J females.

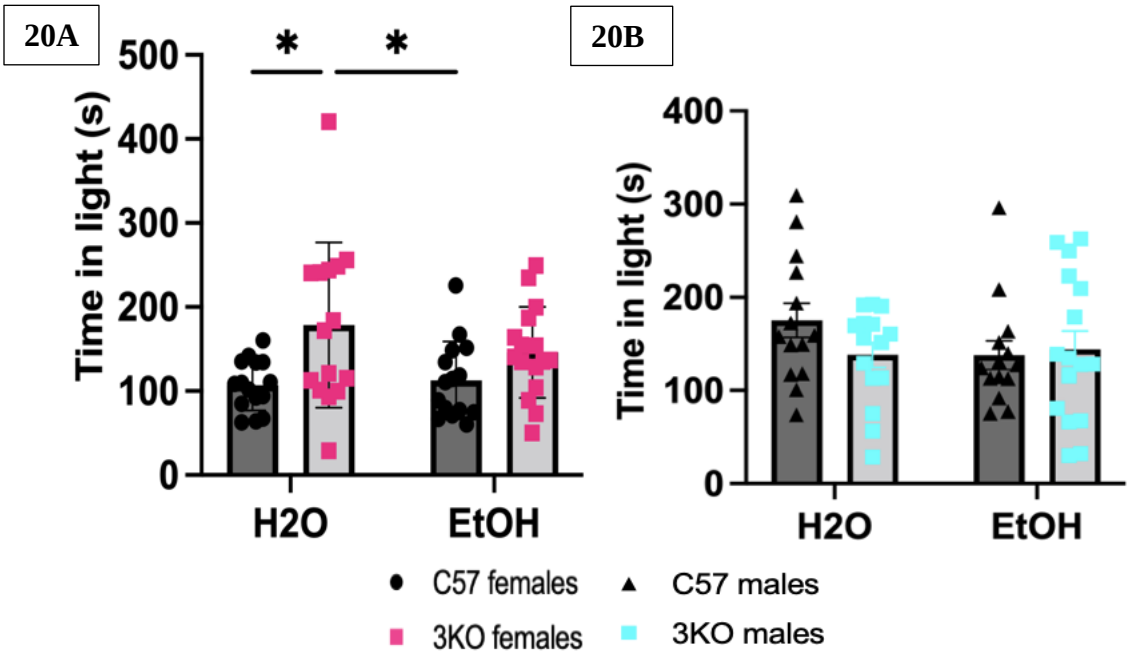


Figure 20: Determining Anxiety-like behavior: light-dark

Time in the light (s) can be used as an indicator of anxiety like behavior. A- Females: time in the light (s) H_2O and EtOH exposed C57BL/6J (H_2O+C57BL/6J and EtOH+C57BL/6J; n=15), and

3KO (3KO+H_2O, 3KO+EtOH; n=15-16). B- Males: time in the light (s) for H_2O and EtOH exposed C57BL/6J (C57BL/6J+H_2O, C57BL/6J+EtOH; n=14) and 3KO (3KO+H_2O, 3KO+EtOH; n=15-16). All data are shown as mean ± SEM, *$p \leq 0.05$

Can prior AIE influence development of preference for nicotine?

There have been several studies that have shown that adolescent EtOH exposure increases the likelihood for substance abuse in adulthood (Grant & Dawson, 1997; Jackson et al., 2002; Salmanzadeh et al., 2020; Waeiss et al., 2019). Therefore, we investigated the effect of prior AIE or H_2O exposure and genotype on the preference for nicotine. Our data reveal no significant treatment effects, showing prior AIE or H_2O exposure does not influence preference for nicotine in this model. Interestingly, the female H_2O ANOVA reveals a genotype effect ($F_{(1,25)}$=6.228, p=0.0195), with no significant solution (saline vs nicotine) effect ($F_{(1,25)}$=0.2029, p=0.6563) , while post-hoc analysis reveals a significant increase in preference for nicotine in H_2O-nicotine exposed 3KO females compared to the H_2O-nicotine exposed C57BL/6J females (p=0.0177) (Figure 21A). The female EtOH ANOVA reveals a genotype effect ($F_{(1,25)}$=5.117, p=0.0326), while no significant differences were found for solution effect ($F_{(1,25)}$=0.5761, p=4549), with post-hoc analysis revealing no further significant differences (Figure 21B). The male H_2O ANOVA and post-hoc analysis reveal no significant differences for genotype ($F_{(1,26)}$=0.02897, p=0.8662), and solution ($F_{(1,26)}$=0.3059, p=0.5849) (Figure 21C). While the male EtOH ANOVA reveals a solution effect (saline vs nicotine) ($F_{(1,24)}$=7.031, p=0.0140), with no further differences found among genotype effect ($F_{(1,24)}$=0.7105, p=0.4076) and in the post-hoc analysis (Figure 21D). Our conditioned place preference (CPP) data continues to reveal a unique genotype and treatment differences as our H_2O-nicotine exposed 3KO females have significantly greater preference for nicotine compared to the H_2O-nicotine exposed C57BL/6J

females. Further, though post-hoc analysis does not reveal significant differences the ANOVA shows further genotype (EtOH-nicotine exposed 3KO vs EtOH-nicotine exposed C57BL/6J females) and treatment (H_2O-nicotine exposed 3KO vs H_2O-saline exposed 3KO males) effects. Overall, our data shows that removal of TNF-α, C1q, IL-1-α increases the preference for nicotine irrespective of prior EtOH exposure. Further, it brings about questions as to the role of this pathway in reward motivated behavior.

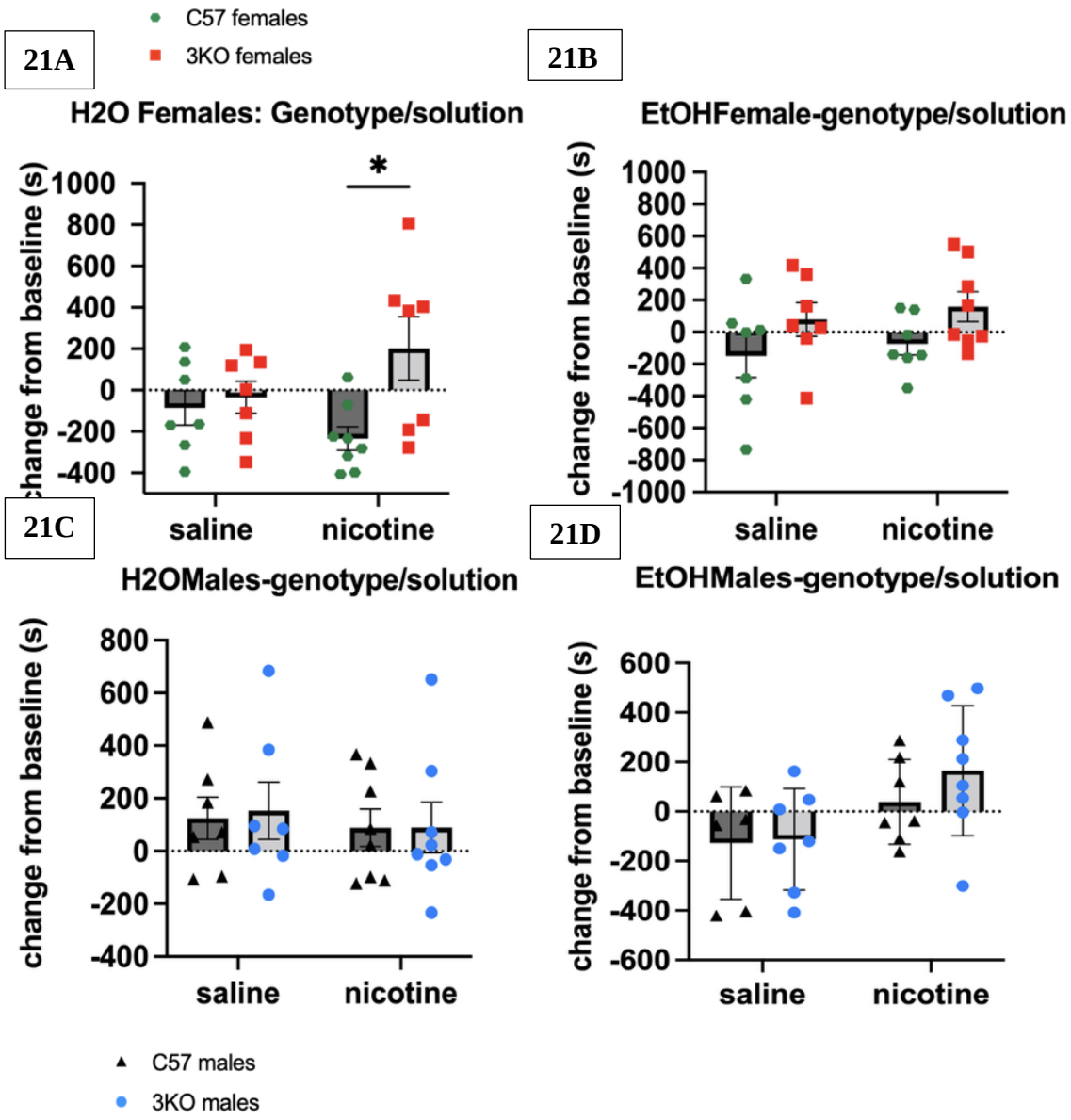


Figure 21: Can genotype and prior EtOH exposure influence preference for nicotine

CPP for EtOH and H_2O exposed C57BL/6J and 3KO male and female mice. A- Females: CPP for H_2O exposed C57BL/6J and 3KO mice (C57BL/6J+H_2O-saline, 3KO+H_2O-saline, C57BL/6J+H_2O-nicotine, 3KO+H_2O-nicotine; n=7-8). B-Females: CPP for EtOH exposed C57BL/6J and 3KO mice (C57BL/6J+EtOH-saline, 3KO+EtOH-saline, C57BL/6J+EtOH-nicotine, 3KO+EtOH-nicotine; n=7-8). C- Males: CPP for H_2O exposed C57BL/6J and 3KO mice (C57BL/6J+H_2O-saline, 3KO+H_2O-saline, C57BL/6J+H_2O-nicotine, 3KO+H_2O-nicotine; n=7-8). D-Males: CPP for EtOH exposed C57BL/6J and 3KO mice (C57BL/6J+EtOH-saline, 3KO+EtOH-saline, C57BL/6J+EtOH-nicotine, 3KO+EtOH-nicotine; n=6-8). All data are shown as mean ± SEM. *$p \leq 0.05$.

Does prior exposure to AIE, and nicotine or saline as well as genotype influence expression of neuroimmune factors in the CA1 of the hippocampus?

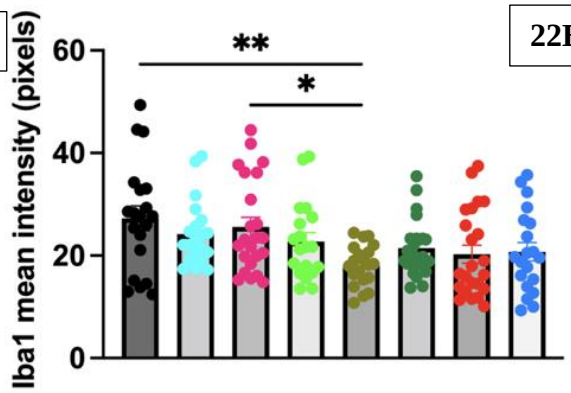
We assessed changes in astrocyte and microglia expression as our model involved the deletion of three genes involved in astrocyte activation. Glial fibrillary acidic protein (GFAP) and ionized calcium binding adapter molecule-1 (Iba1) are two of the most common markers for reactive astrocytes and microglia respectively (Liddelow & Barres, 2017; Ohsawa et al., 2004). Studies have shown EtOH-induces changes in Iba1 and GFAP expression. Qin and Crews (2012) show that C57BL/6J mice intermittently exposed EtOH (i.g.) for 10 days have increased Iba1 and GFAP immunoreactivity as well as increased cell size and altered shape in the cortex, dentate gyrus. Further, a study by Risher and colleagues (2015) shows increased mean astrocyte volume and branch area in the hippocampus of adult rats exposed to AIE from PND 30-46. In order to asses how our model would affect GFAP and Iba1 expression, we assessed mean intensity of GFAP and Iba1, irrespective of sex, and as it relates to sex. First, we assessed Iba1

expression across the various treament groups irrespective of sex, the ANOVA reveals a treatment effect ($F_{(7,160)} = 3.132$, p=0.0040). Post-hoc analysis reveals a significant decrease in Iba1 expression in the the H_2O-saline exposed 3KO mice compared to H_2O-saline exposed C57BL/6J mice (p=0.0063), and in the H_2O-saline exposed 3KO mice compared to the EtOH-saline exposed C57BL/6J mice (p=0.0406) (Figure 22A). The ANOVA assessing sex differences reveals an interaction effect ($F_{(7,152)}$=8.030, p<0.0001), sex effect ($F_{(7,152)}$= 4.343, p=0.0388), and genotype-treatment-solution effect (EtOH/H_2O/Nicotine/Saline) ($F_{(7,152)}$=4.000, p=0.0005). Post-hoc analysis reveals a number of differences in Iba1 expression which can be found in table 1; however, for clarity the graph only displays significant differences for males vs females (Table1/ Figure 22B). When assessing $Iba1^+$ cells irrespective of sex in the CA1 of the hippocampus, the ANOVA reveals a treatment effect ($F_{(7,160)}$=2.243, p=0.0334), while post-hoc analysis reveals a significant decrease in $Iba1^+$ cells in the H_2O-saline exposed 3KO mice compared to the EtOH-nicotine exposed C57BL/6J mice (Figure 22C). For GFAP intensity irrespective of sex, the ANOVA reveals a treatment effect ($F_{(7,160)}$=5.252, p<0.0001), while post-hoc analysis reveals a significant decrease in GFAP expression in the H_2O-nicotine exposed 3KO mice mice compared to the H_2O-nicotine exposed C57BL/6J (p<0.0001), in the the EtOH-nicotine exposed 3KO mice mice compared to the H_2O-nicotine exposed C57BL/6J (p=0.0078), and in the H_2O-nicotine exposed 3KO compared to the EtOH-nicotine exposed C57BL/6J mice mice (p=0.0006) (Figure 22E). For sex differences, the ANOVA reveals a significant genotype-treatment-solution effect (EtOH/H_2O, Nicotine/Saline) ($F_{(7, 152)}$=5.209, p<0.001), with no significant treatment effect ($F_{(1,152)}$=0.2154, p=0.6432). Post-hoc analysis reveals a significant decrease in GFAP expression in the male H_2O-nicotine exposed 3KO mice compared to the male H_2O-nicotine exposed C57BL/6J mice (p=0.0146) (not shown), in the male H_2O-nicotine exposed 3KO mice compared

to the male EtOH-nicotine exposed C57BL/6J mice (p=0.0032) (not shown), in the female H_2O-nicotine exposed 3KO mice compared to the male EtOH-nicotine exposed C57BL/6J mice (p=0.0270), in the male H_2O-nicotine exposed 3KO mice compared to the female H_2O-nicotine exposed C57BL/6J mice (p=0.0034), and in the female H_2O-nicotine exposed 3KO mice compared to the female H_2O-nicotine exposed C57BL/6J mice (p=0.0250) (not shown) (Figure 22F). We assessed the number of $GFAP^+$ cells in the CA1 of the hippocampus, the ANOVA reveals a treatment effect ($F_{(7,160)}$=3.548, p=0.0014), post-hoc analysis reveals a significant decrease in $GFAP^+$ cells in the H_2O-nicotine exposed 3KO mice compared to the H_2O-nicotine exposed C57BL/6J mice (p=0.0014), and in the H_2O-nicotine exposed 3KO mice compared to the EtOH-nicotine exposed C57BL/6J mice (p=0.0013) (Figure 22G). Projection images show the differences between the treatment groups for both Iba1 and GFAP (Figure 22D and 22H). Next, we assessed the influence genotype and treatment irrespective of sex and prior nicotine or saline exposure on Iba1 and GFAP expression. The ANOVA reveals a treatment effect for Iba1 ($F_{(3,164)}$=5.599, p=0.0011), and GFAP ($F_{(3,164)}$=5.489, p=0.0013). While post-hoc analysis for GFAP reveals a significant decrease in GFAP expression in the H_2O exposed 3KO mice compared to the H_2O exposed C57BL/6J mice (p=0.0018) and in the H_2O exposed 3KO mice compared to the EtOH exposed C57BL/6J mice (p=0.0253) (Figure 22I). The Iba1 post hoc analysis reveals a significant decrease in Iba1 expression in the H_2O exposed 3KO mice compared to the H_2O exposed C57BL/6J mice (p=0.0046), and in the EtOH exposed 3KO mice compared to the H_2O exposed C57BL/6J mice (p=0.0130) (Figure 22I). Finally, we assessed sex differences irrespective of prior saline or nicotine exposure, the two-way for Iba1 reveals an interaction effect ($F_{(3, 160)}$ = 15.50, p<0.0001), sex effect ($F_{(1, 160)}$ = 4.955, p=0.0274, and genotype-treatment effect (C57BL/6J/3KO/ H_2O/EtOH) ($F_{(3, 160)}$ = 6.494, p=0.0004) (Figure

22J), while GFAP shows a genotype-treatment effect ($F_{(3, 160)} = 5.464$, p=0.0013), but no sex effect ($F_{(1, 160)} = 0.1949$, p=0.6594), with post-hoc analysis for Iba1 is shown in (table 2/Figure 22J). While GFAP post-hoc analysis reveals no significant differences between groups (Figure 22J). This data continues to reveal interactions (sex, genotype, EtOH, nicotine) that mayinfluence the ability of this astrocyte activation pathway to alter GFAP (astrocyte) expression.

22A



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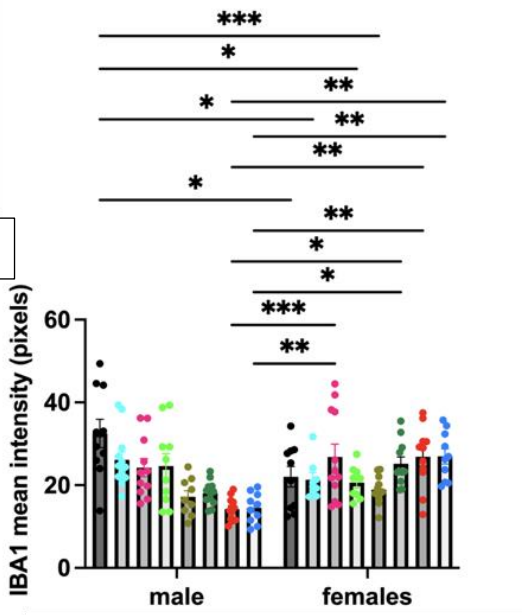

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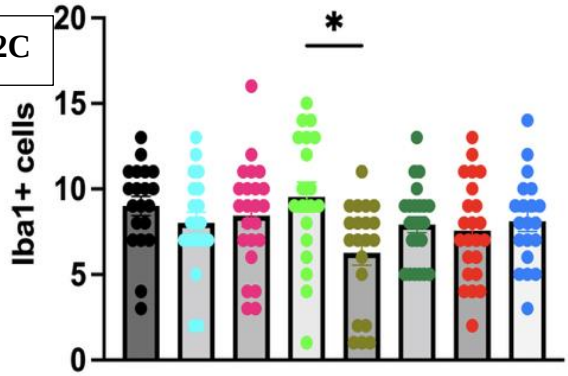

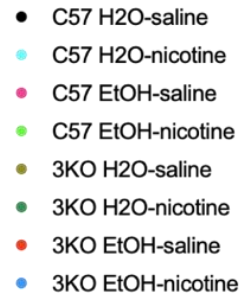

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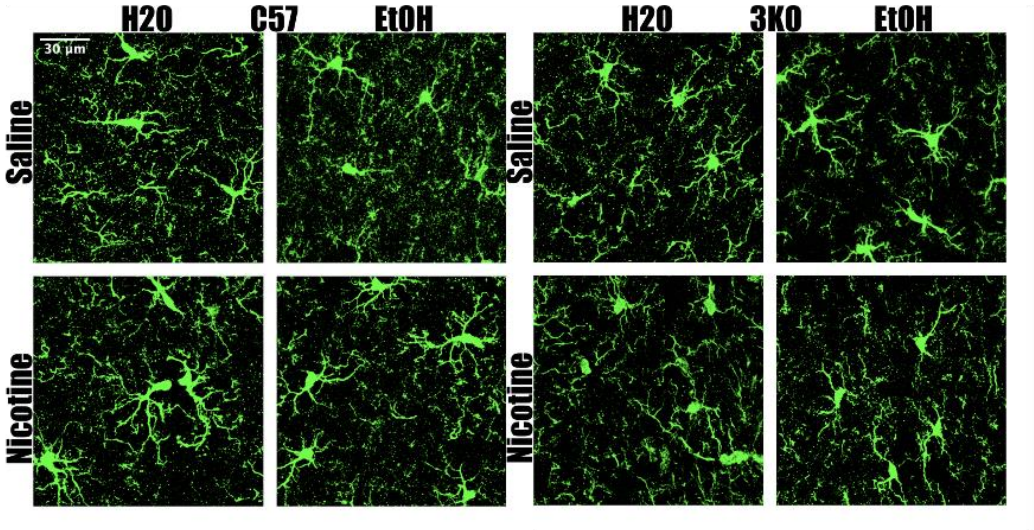
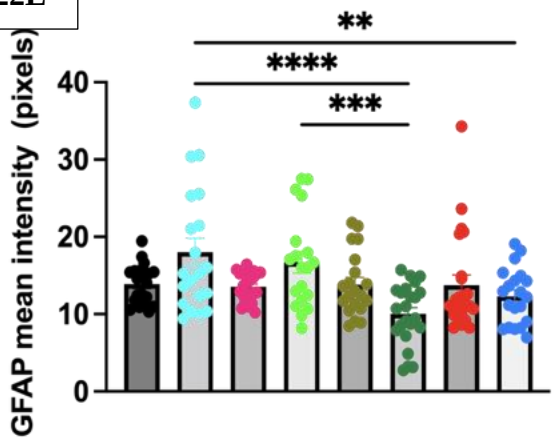


T1

Interaction	Summary (2-way ANOVA with Tukey's post hoc)	P-value	on graph
Male (M): H2O+sal-C57 vs M:H2O-sal-3KO	****	p=0.0002	no
M:H2O+sal-C57 vs M:H2O+nic-3KO	***	p=0.0001	no
M:H2O+sal-C57 vs. M:EtOH+sal-3KO	****	p<0.0001	no
M:H2O+sal-C57 vs M:EtOH+nic-3KO	****	p<0.0001	no
M:H2O+sal-C57 vs F:H2O+sal-C57	*	p=0.0473	yes
M:H2O+sal-C57 vs F:H2O+nic-C57	*	p=0.0436	yes
M:H2O+sal-C57 vs F: EtOH+nic-C57	*	p=0.0149	yes
M:H2O+sal-C57 vs F: H2O+sal-3KO	***	p=0.0008	yes
M:H2O+nic-C57 vs M:EtOH+sal-3KO	**	p=0.0024	no
M:H2O+nic-C57 vs M:EtOH+nic-3KO	**	p=0.0075	no
M:EtOH+sal-C57 vs M:EtOH+sal-3KO	*	p=0.0367	no
M:EtOH+nic-C57 vs M:EtOH+sal-3KO	*	p=0.0311	no
M:EtOH+sal-3KO vs F:EtOH+sal-C57	***	p=0.0009	yes
M:EtOH+sal-3KO vs F:H2O+nic-3KO	*	p=0.0117	yes
M:EtOH+sal-3KO vs F:EtOH+sal-3KO	**	p=0.0012	yes
M:EtOH+sal-3KO vs F:EtOH+nic-3KO	**	p=0.0016	yes
M:EtOH+nic-3KO vs F:EtOH+sal-C57	**	p=0.0030	yes
M:EtOH+nic-3KO vs F:H2O+nic-3KO	*	p=0.0293	yes
M:EtOH-nic-3KO vs F:EtOH+sal-3KO	**	p=0.0040	yes
M:EtOH+nic-3KO vs F:EtOH+nic-3KO	**	p=0.0049	yes

22E



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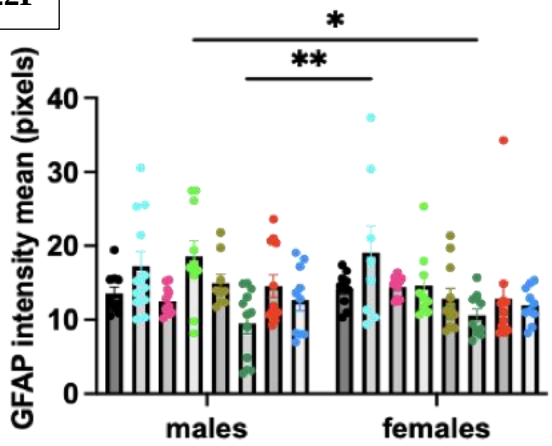

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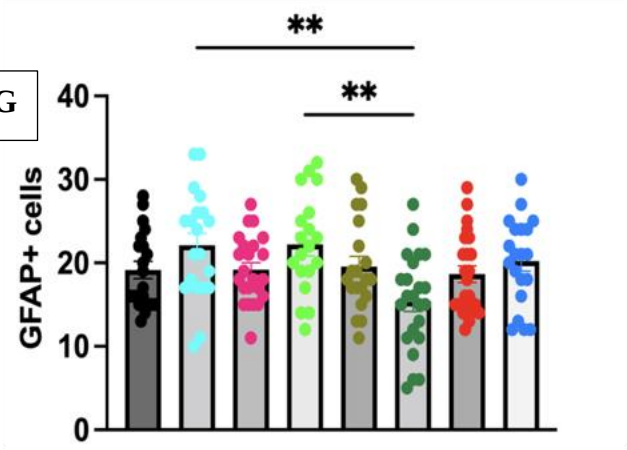

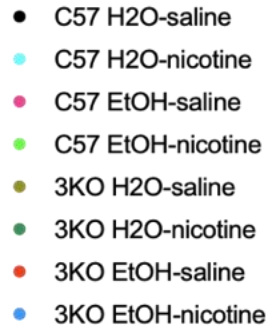

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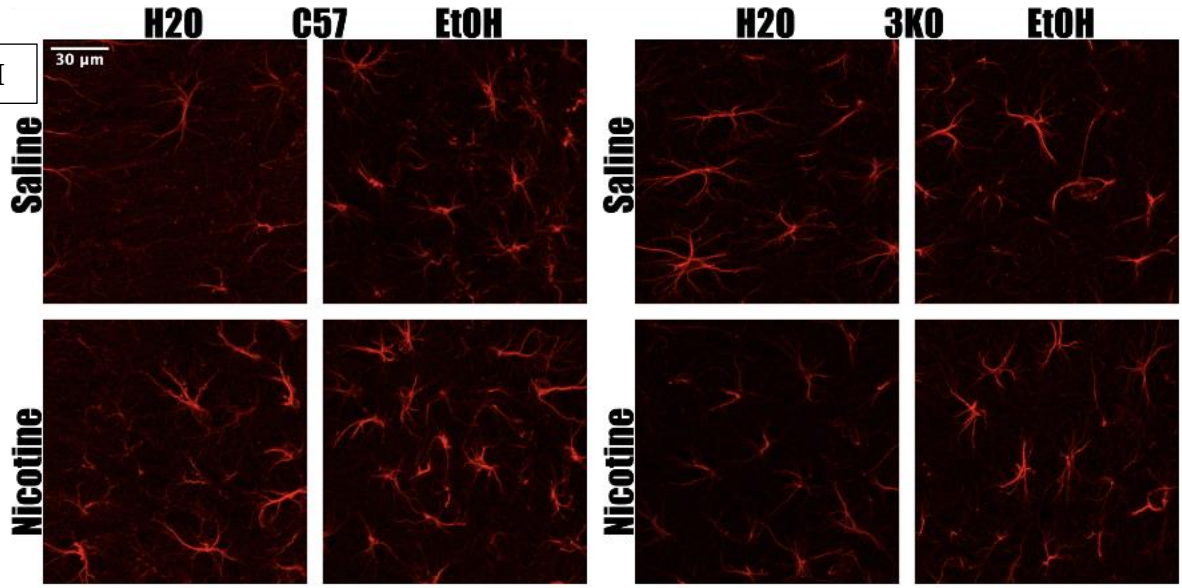

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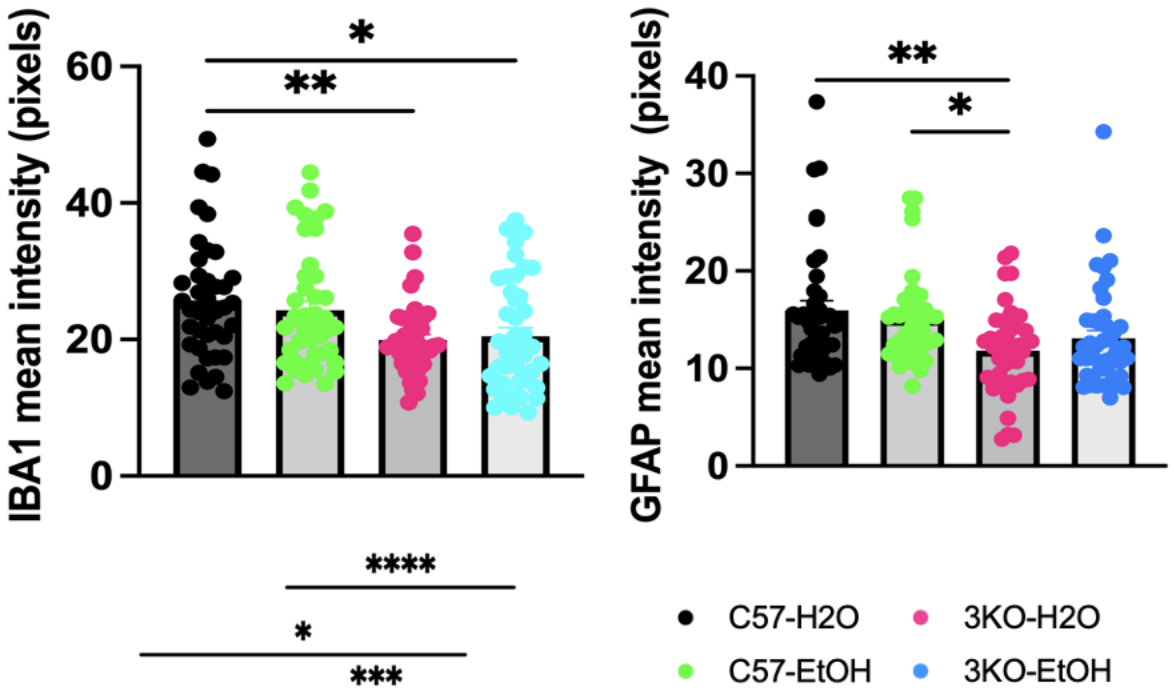

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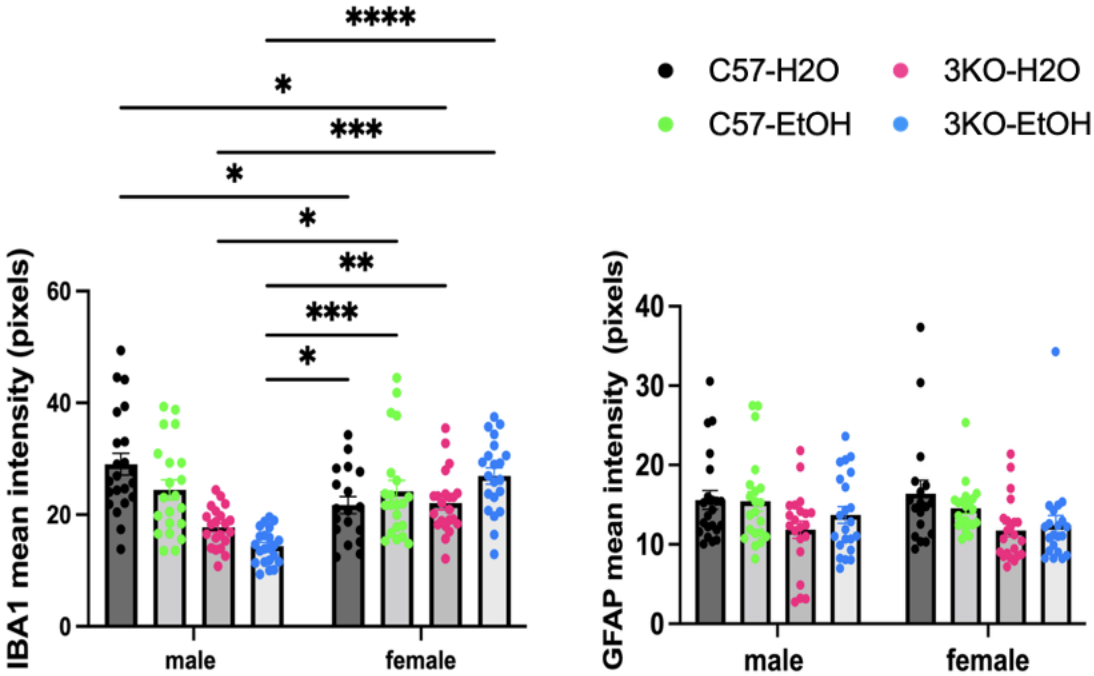

T2

Interaction	Summary (2-way ANOVA with Tukey's hoc analysis)	p-value	on graph
M:C57-H2O vs M:3KO-H2O	****	p<0.0001	no
M:C57-H2O vs M:3KO-EtOH	****	p<0.0001	no
M:C57-H2O vs F:C57-H2O	*	p=0.0204	yes
M:C57-H2O vs F:3KO-H2O	*	p=0.0195	yes
M:C57-EtOH vs M:3KO-H2O	*	p=0.0353	no
M:C57-EtOH vs M:3KO-EtOH	****	p<0.0001	no
M:3KO-H2O vs F:C57-EtOH	*	p=0.0493	yes
M:3KO-H2O vs F:3KO-EtOH	***	p=0.0005	yes
M3KO-EtOH vs F:C57-H2O	*	p=0.0188	yes
M:3KO-EtOH vs F:C57-EtOH	***	p=0.0001	yes
M:3KO-EtOH vs F:3KO-H2O	**	p=0.0059	yes
M:3KO-EtOH vs F:3KO-EtOH	****	p<0.0001	yes

Figure 22: Iba1 and GFAP expression

A- mean Iba1 intensity irrespective of sex. B- sex comparison for mean Iba1 intensity. C- number of $Iba1^+$ cells in the in region of interest within CA1 of the hippocampus. D- representative images for Iba1, scale bar in the upper left corner is 30 μm. **Table 1**: Values from post-hoc analysis for sex differences for mean IBA1 intensity. E- mean GFAP intensity irrespective of sex. F- sex comparison for mean GFAP intensity. G- number of $GFAP^+$ cells in the region of interest within CA1 of the hippocampus. H- representative image for GFAP, scale bar in the upper left corner is 30 μm. I- genotype and treatment differences as shown as mean intensity for Iba1 and GFAP irrespective of sex and prior nicotine or saline treatment. J- sex differences irrespective of prior saline/nicotine treatment. **Table 2**- Values from post-hoc analysis for sex differences irrespective of prior saline or nicotine exposure. Abbreviations: M- male, F-female, Sal-saline, Nic-nicotine, EtOH-ethanol, H_2O- water, 3KO- triple knockout, C57- C57BL/6J. All data are shown as mean ± SEM, *p≤0.05, **p≤0.0078, ***p≤0.0009, ****p≤0.0001.

Our model involves removal of C1q where the binding of its globular heads to the antigen-antibody complex or to apoptotic cells leads to downstream clevage of complement component 3 (C3) (Dunkelberger & Song, 2010; Schartz & Tenner, 2020). The C3 molecule plays a key role in activating and in the continuation of the complement casacde (Ricklin et al., 2016). Further, it is the primary point of convergence of the lectin, classical and alternative complement pathways (Ricklin et al., 2016). Therefore we investiagted the relationship between prior exposure to EtOH and nicotine, as well as genotype on complement component 3 expression in the CA1 of the hippocampus. First we assessed if there is an overall difference in C3 expression irrespective of sex. The ANOVA shows a significant treament effect ($F_{(7,132)}$

=3.368, p=0.0024), while post-hoc analysis reveals a significant decrease in C3 expression in the H_2O-saline exposed C57BL/6J mice compared to the EtOH-saline exposed C57BL/6J mice (p=0.0053), and in the EtOH-nicotine exposed 3KO mice compared to the EtOH-saline exposed C57BL/6J mice (p=0.0086) (Figure 23A). Next we investigated potential sex differences in C3 expression, the ANOVA reveals a sex effect ($F_{(1,126)}$=7.364, p=0.0076), and genotype-treatment-solution effect (EtOH/H_2O/Nicotine/Saline) ($F_{(7,126)}$ = 3.442, p=0.0021). Post-hoc analysis reveals a significant decrease in C3 expression in the EtOH-nicotine exposed 3KO females compared to the EtOH-saline exposed C57BL/6J males (p=0.0242) (Figure 23B). Next, we assessed the influence genotype and treatment irrespective of sex and prior nicotine or saline exposure on C3. The ANOVA reveals a treatment effect for C3 ($F_{(3,136)}$= 3.3961, p=0.0096). Post-hoc analysis reveals significant decrease in C3 expression in the H_2O exposed 3KO mice (p=0.0468) and the EtOH exposed 3KO mice (p=0.0088) compared to the EtOH exposed C57BL/6J mice (Figure 23C). Finally we assessed, sex differences irrespective of prior saline or nicotine exposure, the ANOVA reveals a sex effect ($F_{(1,132)}$= 8.358, p=0.0045), and a genotype-treatment (C57BL/6J/3KO/ H_2O/EtOH) ($F_{(3,132)}$=4.396, p=0.0055). Post-hoc analysis reveals a significant decrease in C3 expression in the EtOH exposed 3KO females compared to the EtOH exposed C57BL/6J males (p=0.0034), and in the H_2O exposed 3KO females compared to the EtOH exposed C57BL/6J females (p=0.0069) (Figure 23D). This analysis reveals C3 expression is increased in the C57BL/6J mice compared to the 3KO mice. These data reveal removal of these three factors, particularly, C1q can effect the C3 expression and other neuroimmune factor expression and may alter the efficacy of the complement pathway.

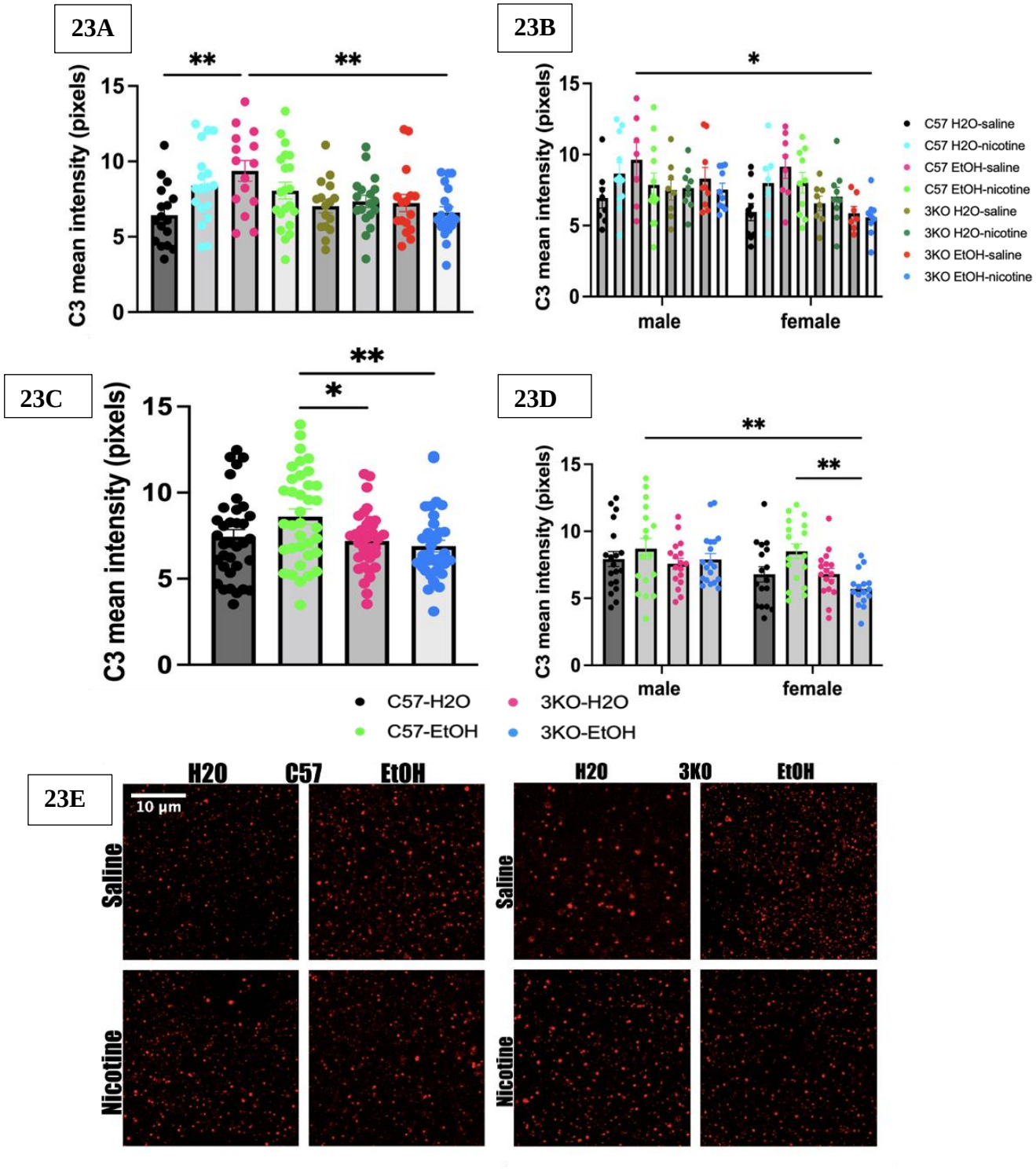

Figure 23: Complement component 3 (C3) intensity

Graphical representation of mean C3 intensity. A- mean C3 intensity within the region of interest (ROI) irrespective of sex (one-way ANOVA). B- sex differences for mean C3 intensity within

the ROI in the CA1 of the hippocampus (two-way ANOVA). C-genotype and treatment differences as shown as mean intensity for C3 (one-way ANOVA). D-sex differences irrespective of prior saline/nicotine treatment (two-way ANOVA). E-Representative images of mean C3 intensity. All data are shown as mean ± SEM, *p≤0.05, **p≤0.0088.

Discussion

The prevalence of adolescent alcohol use and association with later substance use is a major cause for concern in the United States and worldwide. Due to this problem, the short- and long-term effects of AIE has become a major topic of research. Previous studies have shown that EtOH can effect locomotor activity, anxiety, as well as learning and memory (Pandey et al., 2015; M.-L. Risher et al., 2015; Wolstenholme et al., 2017). Further, recent work shows the effects of EtOH are not limited to acute changes but long-term changes which have also been shown to persist into adulthood (M.-L. Risher et al., 2015) and influence subsequent consumption of a rewarding substance. Here, we seek to elucidate the effects of AIE on behavior, future preference for substances of abuse, as well as the underlying mechanism driving these changes.

Effects of genotype and treatment on locomotor behavior

The first interactions we assessed were the effects of AIE, genotype and sex on locomotor behavior. Total distanced traveled and average speed allows us to assess the total ambulation in our animals. Notably, the H_2O exposed 3KO female mice compared to the H_2O exposed C57BL/6J females and the 3KO females compared to the EtOH exposed C57BL/6J show decreased locomotor activity. Though not significant we find a modest decrease in locomotor activity in our EtOH exposed female C57BL/6J and 3KO mice compared to the H_2O exposed C57BL/6J and 3KO female mice (Figure 18). These findings show that locomotor activity is

sensitive to EtOH dose and type/route of administration, and rodent species. In addition to dose-dependent effects, our data reveals genotype effects as the 3KO female mice show hypo-locomotor activity compared to the C57BL/6J mice. This brings about questions as to the role of this microglia derived astrocyte activation pathway in locomotor behavior. Though the entirety of the effects of reactive astrogliosis on locomotor behavior are not known, our findings shed light on the potential role of this microglial-astrocytic activation pathway in locomotor behavior. Further, the data highlights sex differences, as our female data shows more robust difference between the C57BL/6J and 3KO mice. These sex differences are particularly important as female AIE studies are relatively limited compared to male AIE studies, highlighting the need for continued genotype, sex and dose specific studies. Another factor that may contribute to variability in EtOH induced locomotor behavior are the differences in EtOH metabolism between males and females. Previous work has shown that alcohol dehydrogenase (ADH) activity is higher in males than females, which means men are able to metabolize alcohol faster than women, which is why a higher dose is needed in males to achieve similar intoxication levels as females (Chrostek et al., 2003). Based on the genotype and AIE changes in locomotor activity we wanted to investigate if these changes could be attributed to changes in anxiety-like behavior.

The effects of genotype and treatment on anxiety induced locomotor activity

In order to determine if changes in locomotor activity are due to anxiety**,** we assessed time in thigmotaxis of the open field box as well as time in the light of the light dark box. The 3KO mice, regardless of EtOH/H_2O treatment spent less time in thigmotaxis compared to EtOH exposed C57BL/6J females, indicating reduced anxiety in female mice when TNF-α, C1q, IL-1α genes are eliminated. The reduced anxiety observed in the 3KO female mice in open field is further supported by the results of the light-dark assay. The H_2O exposed 3KO females spend

more time in the light compared to the H_2O- and EtOH-exposed C57BL/6J females demonstrating that 3KO female mice have diminished anxiety across multiple assessments. These data suggest that the hypo-locomotor activity observed in the 3KO females in open field may be attributed to decreased anxiety-like behavior.

Although we do not see an EtOH effect in these assays, it is important to note that studies show there is a correlation between adolescent alcohol use and anxiety in adulthood (Crews et al., 2016; Salmanzadeh et al., 2020). Further, alcohol use disorders and anxiety disorders are common comorbidities as alcohol use can lead to anxiety and anxiety can lead to alcohol use (Kushner et al., 2000), highlighting the reciprocal nature of alcohol intake and changes in anxiety-like behavior. Additionally, it has been documented that alcohol, stress, locomotion, and anxiety-like behaviors can be related to one another (Silberman et al., 2009). For example Silberman et al., (2009) highlight the role of corticotrophin-releasing factor (CRF) signaling that may potentially influence anxiety, stress and EtOH administration. This finding is important as CRF mediates the stress response (Jiang et al., 2019). Interestingly, release of cytokines such as IL-6, TNF-α, and IL-10 in response to stress can induce CRF secretion (Jiang et al., 2019; Kariagina et al., 2004). It is important to note that TNF-α is pro-inflammatory. IL-10 is anti-inflammatory and IL-6 can be either pro- or anti-inflammatory, showing that CRF can be induced by various types of inflammatory responses. Highlighting the importance of assessing the expression of various pro- and anti-inflammatory factors in order to determine the primary cause of CRF activation. Our experimental results bring about the question if these three microglia released, astrocyte activating factors may also be involved in anxiety/ stress pathways as the 3KO appear to have decreased anxiety-like behavior. It has been established that factors such as TNF-α, IL-1, and other cytokines involved with inflammation are associated with

anxiety, therefore removal of these three factors may lead to decreased anxiety (Vida et al., 2014). Further, Kariagina and colleagues (2004) using qPCR and northern blots analysis of leukemia inhibitory factor (LIF) and corticotropin releasing hormone CRH knockout mice exposed to lipopolysaccharide (LPS) show that cytokines particularly TNF-α can induce CRF signaling and inflammation. Our model involves removal of three factors including TNF-α, potentially leading to the decrease cytokine levels and decreased CRF signaling in our 3KO mice. While the C57BL/6J mice may have increased cytokine release in response to EtOH and EtOH-induced anxiety it in turn may lead to increased CRF signaling, heightened levels of stress and increased inflammation. Future work investigating cytokine, CRF, and cortisol (stress hormone) levels at this timepoint may help to shed light on the reciprocal interactions between stress, anxiety, and inflammation following EtOH exposure.

The relationship between AIE and preference for nicotine

Alcohol and nicotine are two of the most common drugs of abuse and are commonly co-used (Cross et al., 2017; Funk et al., 2006). Moreover, adolescent alcohol use is a predictor of continued/future use of nicotine (Jackson et al., 2002). To determine the role of AIE and the deletion of TNF-$\alpha^{-/-}$, IL-1$\alpha^{-/-}$, C1q$^{-/-}$ on subsequent reward sensitivity to nicotine CPP was conducted. These data show that H_2O-exposed 3KO females have a greater preference for nicotine compared C57BL/6J females. It is important to note that our data does not show a significant EtOH effect, despite this, our results lead to questions about the role of these three factors in the reward pathway. In order to understand the influence of TNF-α, IL-1α, and C1q on reward, it is important to understand the interactions between alcohol and nicotine. To date many previous studies have focused on nicotine use prior to alcohol use and/or the use of alcohol and nicotine at the same time (Hurley et al., 2012; Tizabi et al., 2007). The studies which assess AIE

prior to nicotine use or preference have mixed findings. For example a study by Boutros et al. (2016), found male Wistar rats who were exposed to 5g/kg of EtOH 3x per day did not consume more nicotine compared to alcohol naïve mice, showing a decrease in sensitivity to nicotine following AIE. While a study by Waeiss et al. (2019) shows that adolescent female alcohol preferring rats who were later exposed to nicotine resulted in the upregulation of alpha-7 ($\alpha 7$) nicotinic receptors with increased nicotine sensitivity and ultimately cross sensitization compared to adult alcohol preferring rats and alcohol naïve rats. One potential underlying factor that may influence the varying results are sex differences since Boutros et al. (2016) uses males and Waeiss et al. (2019) uses females. Previous work in humans has shown that females metabolize nicotine faster than males. In addition to gender and race differences, sex specific hormones such as estrogen can accelerate nicotine metabolism can all influence metabolism (Benowitz et al., 2006). Further, the opposing findings that show that AIE can lead to increased sensitivity to nicotine or no change in preference depending on the strain, dosage, and frequency, and highlight the need for further AIE and nicotine studies in order to fully elucidate the mechanisms driving potential nicotine preference or aversion. Recently there has been a link between inflammation, stress and reward sensitivity, correlating increased stress levels, increases in proinflammatory interleukin-6 (IL-6) and high reward drive in humans (Moriarity et al., 2020). Suggesting that as the propensity to seek a reward increases so too may the activation of stress and inflammation pathways. Alcohol is an inflammatory stimulus, it can also lead to stress/anxiety and has been noted in the work of others (Pohorecky, 1981, 1991; Wang et al., 2010). Interestingly, our data does not show an EtOH effect on anxiety-like and reward motivated behavior there may be a plausible explanation for our results. Much of the AIE work has been done in rats which show some of the same developmental sensitives to EtOH as human

adolescents (Squeglia et al., 2014). It is possible that mice do not have the same developmental sensitivity to EtOH as humans or rats. Further work investigating the potential differences in rat and mouse EtOH sensitivity will be important in understanding the interactions between EtOH-induced stress, anxiety and inflammation. Similarly, it may be plausible that EtOH may not have a direct effect on behavior but may be influencing the function of factors involved in inflammation such as IL-6. Recent work by Moriarity et al. (2020) shows a correlation between IL-6 and reward, which may be the driving mechanisms influencing the appearance of anxiety-like behavior, inflammation, and reward preference in our model. There have been a number of studies investigating anxiety and nicotine (Casarrubea et al., 2015; Johnson et al., 2000); however, studies show that nicotine is commonly used in response to anxiety (Kutlu & Gould, 2015; Ziedonis et al., 2008). Another potential mechanism driving our CPP results may be changes in hippocampal circuitry. The hippocampus is highly important for learning and memory formation, and contains an abundant number of astrocytes and microglia (Bird & Burgess, 2008; Sofroniew & Vinters, 2010). Our model removes three factors involved in microglia (M1)-astrocyte (A1) astrocyte activation but does not affect A2 astrocyte activation. A1 astrocytes are deemed as "neurotoxic" as they lead to release of pro-inflammatory factors and can lead to cell death through NF-κB pathway (Liddelow & Barres, 2017). A2 astrocytes are deemed as "restorative" as they promote neuronal survival, and synaptic repair through the astrocytic release of various signaling factors such as thrombospondins (Eroglu et al., 2009; Liddelow & Barres, 2017). Work by Risher and colleagues (2015) has found that in male rats, AIE leads to chronic upregulation of astrocyte secreted thrombospondins indicating the persistence of A2 activation into adulthood (M. L. Risher et al., 2015). Given the role of A2 activation and the importance of thrombospondin release for synaptogenesis, these data suggest

that astrocytes may be critically important for new synapse formation and synaptic remodeling following AIE. Russo et al. (2010) notes that these newly formed synapses can become mature and integrate into the existing circuitry, increasing receptor expression, which may drive the motivation to seek a drug/reward. Since our genetically modified mouse model only disrupts the A1 pathway and does not disrupt the A2 activation pathway involved in synaptic remodeling, it may be plausible that we are seeing a shift towards A2 astrocyte activation and away from the A1 activation due to the elimination of the three genes. This 3KO-dependent shift from A1 to A2 would drive increased potential for synaptic remodeling in response to repeated nicotine-exposure during the 10-day CPP conditioning period. This is consistent with Aryal et al. (2021) who found nicotine induced time-dependent remodeling of astrocytic processes as well as lengthening of the processes in culture, which may suggest morphometric changes in response to astrocyte activation. This study also found nicotine induced a slight increase in GFAP expression at high nicotine levels (10 μM) but not low nicotine levels (0.1 and 1 μM), further, they found no increase in cytokine levels at any nicotine dose, showing nicotine does not induce reactive astrogliosis (Aryal et al., 2021). This finding suggests that our 3KO mouse model could be strengthening nicotine-induced A2-dependent synaptic remodeling through the elimination of A1 activation; driving increased nicotine preference and reward motivated behavior (Russo et al., 2010). Further, nicotine induced remodeling may also condition the reward pathway by acting through and increasing the amount of nicotinic acetylcholine receptors (nAChRs, $\alpha 4\beta 2$, $\alpha 7$), potentially altering reward preference following the CPP conditioning period in our model. Future studies linking nicotinic receptor expression in areas involved in the reward pathway (ventral tegmental area (VTA), nucleus accumbens (NAc), prefrontal cortex (PFC), amygdala and the hippocampus), its effects on astrocytes, and electrophysiological differences following

nicotine and EtOH exposure may shed light on the how these addictive substances may alter the reward circuitry and synaptic remodeling (De Biasi & Dani, 2011). Additionally, future work investigating the differences in synapse number in our 3KO vs C57BL/6J before and after CPP may elucidate the relationship between nicotine preference and 3KO-dependent strengthening of synaptic circuitry within the reward pathways. Further, western blot analysis for A1 and A2 specific factors, neurotrophic factors, as well as synaptic factors like thrombospondins will shed light on the underlying mechanisms. Additionally, though this study is investigating the preference for nicotine following EtOH exposure, investigating the relationship between stress levels around the time of nicotine administration and after may reveal an important relationship between stress and negative association with a rewarding substance.

These results bring about a number of questions, most of which point towards how these three factors are involved in anxiety and the reward pathway? There are several pathways and factors that may be driving the noted changes; however, a potential area of commonality is the relationship between stress, anxiety, inflammation and reward. Alcohol can be used as an inflammatory stimulus which in turn activates microglia which release TNF-α, IL-1α, and C1q (Kane & Drew, 2016). These three factors activate astrocytes leading to neurodegeneration and neurotoxicity (Liddelow & Barres, 2017). In our model, removal of these three factors appears to decrease anxiety-like behavior and increase preference for nicotine. Our 3KO female mice show decreased anxiety and increased preference for nicotine. This points to a potential relationship between TNF-α, IL-1α, and C1q, anxiety, and reward. The converging points potentially being stress and inflammation. Alcohol can cause and is consumed under stress (Pohorecky, 1981, 1991). Alcohol also causes inflammation, while inflammation can in-turn cause and be triggered by stress (Liu et al., 2017). Further, recent reviews highlight the comorbidity between nicotine

and anxiety, showing that nicotine leads to anxiety; however, it is important to note that some findings are mixed and may be dependent on anxiety disorder (humans) or dose and exposure paradigm used (animal studies) (Kutlu & Gould, 2015). Additionally, previous work implicates nicotine and stress; showing nicotine can increase corticosterone release in rabbits, however, nicotine is commonly used in response to stress (Morse, 1989). There are gaps in knowledge particularly surrounding stress levels following adolescent alcohol exposure and whether that is the driving force behind the noted increased likelihood for substances of abuse in adulthood. This highlights the importance for studies that continue to elucidate many of these complex interactions. Anxiety and stress are triggered by the sympathetic nervous system, which alcohol can trigger and exaggerate the sympathetic nervous systems activity leading to more stress/anxiety and increased cortisol (stress hormone) release (Bystritsky & Kronemyer, 2014). Excessive inflammation has been linked to increased stress which can in turn lead to increased cortisol release (Liu et al., 2017). In terms of reward, positive and negative associations can be influenced by stress and inflammation (Moriarity et al., 2020). As it relates to our study we postulate that if increased levels of stress and neurodegenerative inflammation are present, the drive to seek reward may decrease and the associations with the reward may be negative (Vichaya & Dantzer, 2018). The differences in preference for nicotine in the control C57BL/6J and 3KO female mice in the CPP assay may be due to changes in anxiety and/or stress around the time a rewarding substance. Our mice are tested for anxiety a few days prior to the beginning of CPP, it may be plausible that control mice are still displaying anxiety-like behavior during the first few nicotine conditioning sessions. This in turn may lead to negative associations, as the initial memory is formed at a time when anxiety and stress are heightened. However, when less stress and neurodegenerative inflammation are present, the drive to seek the reward may increase

while the association with the reward are positive. This theory may explain the why our C57BL/6J mice do not show increased preference for nicotine while our 3KO mice have decreased anxiety and increased reward sensitivity compared to the C57BL/6J controls. Each of these processes seem to have reciprocal interactions which is why it is plausible that interactions stress, anxiety, inflammation, and reward are driving our results. Our 3KO mice show a preference for nicotine, therefore the removal of these three factors which are all implicated in inflammation and cell death appears to lead to positive correlations with nicotine preference and positive memory association with the associated reward and environment that the reward is given.

Can AIE, sex and genotype alter Iba1, GFAP, and C3 expression?

Given that our model is specifically eliminating three microglia released factors involved in A1 or neurotoxic astrocyte activation, as well as a factor involved in the activation of the complement cascade we assessed Iba1, GFAP, and C3 expression in our C57BL/6J and 3KO mice. Iba1 has been shown to be highly expressed in ramified (branched/reactive) microglia (Ito et al., 1998). While astrocytes express GFAP under normal conditions; however, its expression increases under injury states where astrocytes become reactive (Liddelow & Barres, 2017). Previous work has shown that adolescent rats exposed to EtOH over a 55-day period (PND 35-90) using a two bottle choice paradigm showing a decrease in GFAP and Iba1 expression with increases in nitric oxide expression (Teixeira et al., 2014). Our data reveals significant effects for Iba1, C3, GFAP irrespective of sex, as well as sex-dependent differences. The Iba1 data reveals a host sex and genotype specific interactions (see Figure 21B and table 1); particularly as the EtOH-nicotine and EtOH- saline exposed 3KO females have higher Iba1 expression compared to the EtOH-nicotine and EtOH-saline exposed 3KO males, highlighting sex specific influences on

microglia expression. When collapsed across saline and nicotine exposure in the CPP task, 3KO females have similar or increased Iba1 expression compared to C57BL/6J females, while the 3KO males decreased Iba1 expression compared to C57BL/6J males have. We also found decreased GFAP expression in the 3KO mice compared to the C57BL/6J mice irrespective of sex, further highlighting genotype effects. When collapsed across sex, our data show increased C3 expression when C57BL/6J mice are exposed to EtOH. Whereas, elimination of TNF-α, IL-1α, and C1q genes attenuated these effects. When assessing sex differences, our data reveals decreased C3 expression in the EtOH-nicotine exposed 3KO females compared to the EtOH-saline exposed C57BL/6J males showing sex, and genotype effects on C3 expression. These findings suggest that in males there is increased activation of pathways that drive C3 expression and that attenuation of EtOH-dependent C3 expression can be achieved through the elimination of TNF-α, IL-1α, and C1q. While removal of these factors seems to lower C3 expression in a sex-dependent and sex-independent manner. GFAP expression shows some sex-dependent and genotype specific effects particularly between the EtOH-nicotine exposed C57BL/6J males compared to the EtOH-nicotine exposed 3KO females. Though there are not as many significant interactions in GFAP expression as Iba1, this may be due to increased GFAP expression in the A2 astrocyte activation pathway in the 3KO mice due to the elimination of A1 reactivity. To further explain, GFAP is expressed in both subtypes of reactive astrocytes A1 and A2, because of this we cannot rule out an increase in A2 expression (T. Li et al., 2019). The lack of significant differences in GFAP expression may be attributed to the time at which our animals are sacrificed (Gaudet & Fonken, 2018). We next assessed sex specific expression of C3, GFAP and Iba1 collapsed across nicotine or saline exposure. This assessment allows us to specifically investigate the relationship between AIE, genotype, and sex. The data show that the 3KO mice

have decreased expression of C3, GFAP, and Iba1 compared to C57BL/6J mice. Interestingly, previous work shows sex specific differences in GFAP expression differences (Marco et al., 2017). An AIE study using a drinking in the dark paradigm with male and female Wistar rats by Marco et al. (2017) shows increased GFAP protein expression in AIE exposed female mice compared to controls while males show decreased GFAP protein expression in the hippocampus compared to controls, while at PND 68. Differences in time point as our mice are sacrificed at PND 85 while theirs is at PND 68 may account for the lack of sex specific differences. Given that our triple knockout mouse model does not eliminate the pathways involved in microglia activation increased Iba1 expression was not surprising; however, the fact that 3KO females have higher Iba1 expression compared to 3KO males suggests that there are fundamental differences in neuroinflammatory response in male versus female mice. As previously mentioned astrocyte expression can be dependent on timepoint, as immediate and acute responses to injury/inflammation involves an immediate inflammatory response while at subacute and chronic stages, glia scar formation as well as synapse and neuronal repair (Gaudet & Fonken, 2018; Liddelow & Barres, 2017).Therefore future work investigating GFAP and Iba1 expression at various timepoints and correlating A1 and A2 specific factors such as TGF-β, S100A10, complement factor B (CFB), and C3 (Gaudet & Fonken, 2018; Liddelow et al., 2017). C3 expression of the complement cascade plays a crucial role in the innate immune systems response to injury, inflammation or disease (Dunkelberger & Song, 2010; Ricklin et al., 2016). The cascade begins with the initiation of three different pathways: classical, lectin, and alternative pathway. The classical pathway is initiated when the globular heads of C1q bind to the antigen-antibody complex or to apoptotic cells (Dunkelberger & Song, 2010; Schartz & Tenner, 2020). The lectin pathway is activated when mannose binding lectin (MBL) and MBL-

associated serine proteases (MASP1 and 2) interact with pattern-associated molecular patterns (PAMPS) carbohydrates (Dunkelberger & Song, 2010; Schartz & Tenner, 2020). Both of these pathways cleave complement 2 (C2) and 4 (C4) forming C3 convertase (C4bC2b) which in turn cleaves C3 (Dunkelberger & Song, 2010; Schartz & Tenner, 2020). The current 3KO model involves the removal of C1q, which will affect the activation of the classical pathway hindering C3 expression, reducing inflammation, opsonization, and cell lysis (Dunkelberger & Song, 2010; Ricklin et al., 2016; Schartz & Tenner, 2020). These studies show the importance of C3 in the complement cascade; however, there are relatively few studies investigating EtOH exposure and C3 expression with even fewer investigating the impact of AIE and nicotine exposure on C3 expression in adulthood. The few studies linking EtOH consumption and C3 primarily revolves around EtOH-induced changes to the liver which Bykov et al. (2006), Pritchard et al. (2007) and Zhong et al. (2019) have investigated.

In summary, our findings shows that elimination of TNF-α, IL-1α, and C1q has a significant impact on glial-immune mechanisms and multiple behavioral outcomes including nicotine reward sensitivity. The elimination of the A1 astrocyte reactivity pathway resulting in a shift towards the A2 astrocyte reactivity, which is heavily involved in neuronal remodeling, could be a critical contributor to the increased nicotine sensitivity that we have observed. Future work characterizing sex differences with regard to the type of microglial and astrocyte reactivity that is occurring during nicotine intake and direct assessment of neuronal remodeling should help elucidate the role of TNF-α, IL-1α, and C1q in reward sensitivity. Additionally, future work investigating the levels of the stress hormone, cortisol, during alcohol exposure, as well as before and after these behavioral assays may be the key to understanding these complex results. Further, investigating the upregulation of inflammatory factors at the same time points as cortisol can

shed light on the relationship between stress, inflammation, and adolescent alcohol exposure. Examining the associations between stress level during the battery of behavioral assays may explain the results of this experiment and may shed light on the relationship between adolescent EtOH exposure, and the acute and long-term effects on stress and inflammation.

Conclusion

While we have little evidence from this study to suggest that microglia-astrocyte signaling is involved in the long-term effects of AIE we have uncovered a novel role for this signaling pathway in reward responsivity to nicotine in female mice that is not replicated in male mice. Deletion of TNF-α, IL-1α, and C1q in female mice reduced anxiety and increased reward sensitivity to nicotine, suggesting a critical role for microglia-astrocyte communication in the modulation of reward. Further work is necessary to determine whether this pathway is critical to multiple drugs of abuse or uniquely suited for modulating nicotine sensitivity. Future work assessing the characteristics of astrocyte activation will be important for determining whether neuronal remodeling of the reward circuitry through astrocyte activation is a contributing factor.

Acknowledgements

We would like to thank Shane Liddelow, Ph.D. for providing the triple knockout mice, Dr. Kelly Hopper, DVM and the animal technician staff for their care and aid in providing a constant and comfortable environment for the animals. We would like to thank Dr. Brandon Henderson and David Neff for their excellent technical assistance.

Author Contributions

The authors confirm contribution to the paper as follows: Study conception and design: C.M.H, M.L.R; data collection; C.M.H., H.G.S., analysis and interpretation of results: C.M.H, H.G.S,

M.L.R, P.J.M; manuscript preparation: C.M.H, M.L.R. Authors C.M.H and M.L.R reviewed the results and approved of the final manuscript.

Funding

This study was funded by the West Virginia Clinical and Translational Science Institute (WVCTSI) Pilot Grant (2U54GM104942-03) (MLR), the Veterans Affairs BLR&D BX005403 and BX002505 (MLR), and the NIH grant P20GM103434 to the West Virginia IDeA Network of Biomedical Research Excellence.

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APPENDIX A: APPROVAL LETTER

MARSHALL UNIVERSITY.
www.marshall.edu

Office of Research Integrity

February 2, 2022

Christian Harris
8 Pyramid Drive, Apt 821
Huntington, WV 25705

Dear Ms. Harris:

This letter is in response to the submitted dissertation abstract entitled *"Application of Confocal Microscopy to Study the Neural Mechanisms Underlying Insect and Rodent Behavior."* After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Institutional Animal Care and Use Committee (IACUC) has reviewed and approved the study under protocol #750 (Dr. Mary-Louise Risher, PI). The applicable human and animal federal regulations have set forth the criteria utilized in making this determination. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and a determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director
Office of Research Integrity

APPENDIX B

ABBREVIATIONS

Ds- trochanteral extensor motorneurons

SETi- tibial extensor

SEM-scanning electron microscopy

CT- computed tomography

CT-joint- coxo-trochanteral joints

NOMPc- no mechanoreceptor potential C

UV- ultraviolet

NIH-National Institutes of Health

Nm- nanometers

diI-1,1’ dioctadecyl-3,3,3’,3’-tetramethylindocarbocyaninae perchlorate

SPSS- statistical package for the social sciences

pCS- proximal campaniform sensilla

aCS- anterior campaniform sensilla

dCS- dorsal campaniform sensilla

fCS- femoral campaniform sensilla

PG1- proximal group 1

AG1- anterior group 1

AG2-anterior group 2

PG2-4- proximal groups 2-4

CS3- campaniform sensilla 3

CS8- campaniform sensilla 8

CS5- campaniform sensilla 5

CS11- campaniform sensilla 11

SD-standard deviation

Mg- milligram

mm- millimeters

SAMHSA- substance abuse and mental health services administration

WHO-world health organization

CNS- central nervous system

PFC-prefrontal cortex

AUD-alcohol use disorder

AIE-adolescent intermittent ethanol

CIE-chronic intermittent ethanol

g/kg- grams per kilogram

EtOH-ethanol

COX-2- cyclooxygenase-2

iNOS- inducible nitric oxide

PND-post-natal day

Hr- hour

Mins-minutes

Hrs-hours

BDNF- brain derived neurotrophic factors

mRNA- messenger ribonucleic acid

Arc protein- activity regulated cytoskeleton-associated protein

CeA-central nucleus of the amygdala

HDAC- histone deacetylases

MeA- medial amygdala

LTP-long term potentiation

PSD95- post-synaptic density marker 95

TSP- thrombospondins

EAAT- excitatory amino acid transporter

GLT-1- glutamate transporter 2

GLAST- glutamate transporter 1

NMDA- N-methyl-D aspartate

STAT3- signal transducer and activator of transcription 3

VEGF- vascular endothelial growth factor

TNFα- tumor necrosis factor alpha

C1q- complement component 1q

IL-1α- interleukin-1 alpha

NF-κB- Nuclear Factor-kappa B cell

IFN-γ- interferon- gamma

TNFR- tumor necrosis factor receptor

TNF-tumor necrosis factor

IL-1R- interleukin-1 receptor

MAPK- mitogen activated protein kinase

PAMP- pathogen-associated molecular pattern molecules

C3- complement component 3

3KO- triple knockout

MOR- μ −opioid receptors

CPP- conditioned place preference

CPA- conditioned place aversion

v/v- volume/volume

Mg/kg- milligrams per kilogram

PFA- paraformaldehyde

PBS- phosphate buffered saline

TBS- tris buffered saline

PBST- phosphate buffered saline-triton X-100

GFAP- glial fibrillary acidic protein

Iba1- ionized calcium binding adaptor molecule 1

IgG- immunoglobulin

DAPI- 4',6 diamidino-2-phenylindole

SEM- standard error of the mean

ANOVA- analysis of variance

m-meters

m/s- meters per second

H2O-water

s- seconds

α7-nicotinic receptor alpha 7

TLR- toll like receptor

IL6-interleukin 6

IL4-interleukin4

IL13-interleukin 13

IL10-interleukin 10

PPAR γ- as proliferator activated receptor gamma

TGF-β- transforming growth factor-beta

SPARC-secreted protein acidic rich in cysteine

TRAF2-TNFR-associated factor 2

p38MAPK- p38 mitogen-activated protein kinase

MKK3-MAPK kinase 3

IKK- inhibitor of NF- κB kinase

TRADD- TNFR1 activates TNFR-associated death domain protein

FADD- Fas associated death domain

IRAK- Interleukin 1 receptor-associated kinase 1

TRAF6- TNF receptor-associated factor 6

i.g.- intragastric gavage

i.p.- intraperitoneal

HMGB1- High- mobility group box 1

RAGE-receptor for advanced glycation end products

MBL- mannose binding lectin

MASP1 and 2- MBL- associated serine proteases 1 and 2

C4bC2b-C3 convertase

PAMPS- pattern-associated molecular patterns

C3bBb- active C3 convertase

C4-complement component 4

C2-complement component 2

VTA- ventral tegmental area

NAc-nucleus accumbens

NeuN-neuronal nuclei

LIF-leukemia inhibitory factor

CRF-corticotropin releasing factor

CRH-corticotropin releasing hormone

ADH-alcohol dehydrogenase

nAChR- nicotinic acetylcholine receptor