NEURAL CONTROL OF MALE AND FEMALE AGGRESSION IN *DROSOPHILA*

Thesis by

Hui Chiu

In Partial Fulfillment of the Requirements for

the degree of

Doctor of Philosophy

CALIFORNIA INSTITUTE OF TECHNOLOGY

Pasadena, California

2021

(Defended April 27, 2021)

Hui Chiu
ORCID: 0000-0002-1820-8411

ACKNOWLEDGEMENTS

I am extremely lucky to be surrounded by kind and inspiring people. The pandemic makes me feel more grateful than ever.

My advisor David Anderson is my biggest inspiration. His enthusiasm for science, the way he thinks about questions, and his ability to design experiments blew my mind. It is as if I wanted to learn how to play tennis, and I magically have Federer as my coach. It always amazes me how he notices everyone's ups and downs in the lab no matter how busy he is, and how he goes out of his way to pull you out of the dumps.

I would not have been able to make this far if not for the assistance and encouragement from my committees. Paul Sternberg rescued me from the spiral of Imposter Syndrome when I started at Caltech. His trust and positivity give me strength to try harder, especially at difficult times. Betty Hong kindly taught me electrophysiology (while setting up her own lab!). I benefited tremendously from her selfless sharing and is deeply inspired by her philosophy of research and education. I simply cannot thank her enough. Viviana Gradinaru always encourages me to dream big and speak up for myself. "Tell people what you want so they know how to help you". The more I practice the more I appreciate her words to me. Markus Meister is like a mirror that reflects the blind spots in my thinking process. He pushes me to get out of my comfort zone, which is exactly what I need!

I also had the privilege to work with the most talented group of people during different stages of my graduate study. Hillel Schwartz, Nick Flytzanis, Ken Chan, and Jennifer Treweek are the best rotation mentors that I could possibly hope for. Their generosity did not stop as my rotation ended; instead they were always there to help no matter if it was my fellowship proposal or conference presentation.

Kiichi Watanabe mentored me through my time in the Anderson lab. I respect and trust him as if he is my brother. Eric taught me everything about fly aggression and behavioral experiments. He is also my source of fun tips, like secret restaurants and pasta recipes. Yonil

Jung provided valuable insights on all my experiments. Shuo Cao taught me how to perform whole brain imaging. Joe Ouadah generously shared his postdoc hunting experiences with me. Ke Ding is my go-to for reagent preparation questions. Kenta Asahina kindly shared flies with me. Barret Pfeiffer shared his tricks for optimizing plasmids. Brian Duistermars shared the best playlist with me and allowed me to use his moving magnet setup. Allan Wang and Hidehiko Inagaki took good care of me while I was at Janelia. They have continuously been such an inspiration to me.

I like how Brady Weissbourd told me to "put it together" whenever I had micro meltdowns in the lab. It is such a valuable lesson to see how he established jellyfish as a new research model from scratch. Moriel Zelikowsky kindly shared her grant application material with me. Ann Kennedy edited my research proposal. Tomomi Karigo is my cheerleader, and kindly took care of Batman when I went to conferences. Zeynep Turan is so chill and fun to be around! She gave me my favorite nail polish. Stefanos Stagkourakis shared his decision-making strategies with me. He is my model for how to commit a hundred percent to both work and life. Amit Vinograd taught me so much about Jewish traditions. Dong-Wook Kim kindly shared his survival guides and convinced me that there will be an end to the tunnel. Lingyun Li listened to my problems about pathfinding patiently and encouraged me not to rush for decisions. George Mountoufaris generously shared his tips on postdoctoral fellowship applications. Bin Yang is the expert on everything, I mean, everything. Mengyu Liu, Kathy Cheung, Jia Shen, and Adi Nair are a breath of fresh air in the lab. Their energy and enthusiasm for science encourage me to keep exploring. My thanks to Gina Mancuso, Celine Chiu, and Xiaolin Da for their huge help and support. Because of their hard work, I never have to worry about administrative hassles.

I learned a tremendous amount from working with Carlos Lois and Ting-Hao Huang. Every discussion with them left me inspired and motivated! Huge thanks to my master advisors, Yi-Chun Wu and Chieh Chang, who continuously provided valuable advices and support.

I want to thank Mike Vondrus and Masako Vondurs from the bottom of my heart. They are like my grandparents who always take good care of me. They show me how to live a happy life with Czech breads, beers, and smoked meats. A huge shout-out to Debra Anderson, who warms up my heart with her smile and kind words every time. My dear friends Hang-Shiang Jiang, Gee-way Lin, Jui-Fan Hsieh, Yi-Ching Liu, Yun Mou, and Wen-Chieh Hsieh have been there for me through all these years. I am forever grateful for my father, mother, uncle, and brother, who love and support me unconditionally.

My husband Jiun-Yann Yu is simply the best thing that happened to me. He understands me better than I know myself and has always believes in me even when I do not. This is the first time that I am confident about where my life is heading because everything is automatically better when he is around.

ABSTRACT

Aggression is essential for an individual's survival, but it can also lead to unfavorable consequences when misregulated. It is thus important to study the neural basis of this behavior not only for learning how the nervous system is constructed to generate an innate behavior but also for finding the causality of misregulation. Although many circuit and molecular mechanisms underlying aggression have been revealed, our knowledge is mostly restricted to males. Given that sexual differences in aggression are seen in most if not all species, the mechanisms that we learned in one sex may not be directly applied to the other. Therefore, studying the neural basis of aggression in both sexes is necessary for gaining a full understanding of this behavior. *Drosophila* serves as a unique model for such studies because males and females differ not only in the level of aggressiveness but also in the motor patterns. Interestingly, the aggression-promoting neurons that have been identified so far are mostly sex-specific, raising the possibility that males and females adopt distinct circuits for controlling aggression. However, many sexually shared features of aggression also imply the existence of common circuit elements. My thesis work investigated whether any aggression circuit modules are shared by the two sexes and how the circuit is organized to generate sexually shared and dimorphic motor patterns. Through a behavioral screen and the genetic intersection approach, we identified a pair of sexually shared neurons, CAP, that regulates aggressive approach in both sexes, as well as a pair of male-specific neurons, MAP, whose activation promotes the transition from approach to male-specific attack. We subsequently identified the female homologue, fpC1 neurons, whose activation induces female aggression. Supported by the *in vivo* imaging and the behavioral epistasis results, we confirmed the functional connectivity between CAP and MAP/fpC1 in males and females, respectively. Lastly, we showed that the connectivity between CAP and MAP/fpC1 is strengthened in socially isolated flies, which exemplifies how circuits can be modified by social isolation to enhance aggression in both sexes. The connectivity between CAP and MAP/fpC1 provides a circuit logic for the control of sexually shared and dimorphic aggressive behaviors. It can be used as an entry point for circuit mapping as well as for further investigation of mechanisms underlying sexual differences in aggression.

PUBLISHED CONTENT AND CONTRIBUTIONS

Chiu, H., Hoopfer, E.D., Coughlan, M.L., Pavlou, H.J., Goodwin, S.F., and Anderson, D.J. (2021). "A circuit logic for sexually shared and dimorphic aggressive behaviors in *Drosophila*". Cell *184*, pp. 507-520. doi: 10.1016/j.cell.2020.11.048

H.C. participated in the conception of the project, performed the experiments, analyzed and prepared the data, and co-wrote the manuscript.

Jung, Y., Kennedy, A., Chiu, H., Mohammad, F., Claridge-Chang, A., and Anderson, D.J. (2020). "Neurons that function within an integrator to promote a persistent behavioral state in *Drosophila*". Neuron *105*, pp. 322-333. doi: 10.1016/j.neuron.2019.10.028

H.C. generated the reagents.

Watanabe, K., Chiu, H., Pfeiffer, B.D., Wong, A.M., Hoopfer, E.D., Rubin, G.M., and Anderson, D.J. (2017). "A circuit node that integrates convergent input from neuromodulatory and social behavior-promoting neurons to control aggression in *Drosophila*". Neuron *95*, pp. 1112-1128. doi: 10.1016/j.neuron.2017.08.017

H.C. performed the functional imaging experiments, analyzed and prepared the data.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS AND/OR TABLES

Chapter 1

Chapter II

Chapter 1

INTRODUCTION

The purpose of this chapter is to give you an overview of my thesis, which centers on the neural control of aggression in *Drosophila*. The first chapter is to provide you with the background information by walking you through a series of questions: why I study aggression, what were the knowledge gaps, and, importantly, why I chose to study the neural mechanisms underlying the sexual differences in fly aggression? The second chapter is to demonstrate my findings, which delineate a circuit motif that controls the common and sexually dimorphic aggressive behaviors in both sexes of flies. The last chapter contains a list of questions that remain to be addressed. These questions, in my opinions, are better studied in *Drosophila* than other species given the recent technical advances. I also list several tools that I think would push the boundaries of neuroscience research in flies.

Overview of aggression

Why do we study aggression? Being one of the most evolutionarily conserved behavior, aggression is like a double-edged sword. For individuals, aggression is essential for survival. Animals display aggression to secure territory, food, or mating opportunities. When necessary, aggression is also used to protect one's own or offspring's safety against predators or intruders. However, uncontrolled aggression is problematic. Violence in different forms takes a heavy toll in human society especially. Oftentimes pathological aggressive behaviors are the outlets of various psychological disorders, such as schizophrenia and bipolar disorder (Kohn and Asnis; Lane et al., 2011). Therefore, it is important to study how aggression is encoded in the brain not only for the reasons of understanding the neural mechanisms underlying social behaviors, but also for the purposes of obtaining references for therapeutic applications.

Aggression has been a research focus for more than 70 years (Hashikawa et al., 2017; Kravitz and Huber, 2003). Much progress has been made to identify the circuitry that governs

aggression and to characterize the range of the behavior in both rodents and flies (Asahina, 2018; Hashikawa et al., 2018; Hoopfer, 2016; Wei et al., 2021). At the structural level, our understanding about the aggression circuitry in rodents has expanded extensively beyond the loosely defined "hypothalamic attack area" identified in the early electrical stimulation studies (Siegel and Skog, 1970). Various sub-hypothalamic structures, including ventromedial hypothalamic nucleus, lateral septum, medial amygdala, and ventral premammillary nucleus, have been shown to regulate aggression in an array of subsequent works (Lee et al., 2014; Stagkourakis et al., 2018; Unger et al., 2015; Wong et al., 2016). The functional connectivity between these nuclei and other brain regions also serves as a physical reference for further mapping of the circuitry. Studies in flies, on the other hand, provide finer details about the genetic or cellular mechanisms underlying aggressive behaviors (Hoopfer, 2016; Zwarts et al., 2012). Circuit studies in the two systems well complement each other.

At the behavioral level, aggression in both rodents and flies is shown to be more than just an array of fixed motor patterns that can be triggered by sensory cues. Instead, many observations have documented the short-term or long-term 'plasticity' in this behavior; that is, animal's aggressiveness is clearly shaped by both external and intrinsic factors, for examples, defeat experience or reproductive cycles (Laredo et al., 2014; Penn et al., 2010; Takahashi et al., 2017). Presumably the behavioral plasticity is derived from changes in the neural circuitry. It is therefore reasonable to assume that studies on how such plasticity is implemented at the circuit level to influence animals' aggressiveness can be pursued in parallel with the on-going mapping of the circuitry.

While we are moving toward these goals, I would argue that an important biological variable has been long ignored in most of the studies: sex. Like many other innate behaviors that are related to reproduction, aggression is sexually dimorphic in most if not all species that display this behavior (Asahina, 2018; Hashikawa et al., 2018; Tieger, 1980). This means that what we learn about the neural control of aggression from one sex cannot be automatically applied to the opposite sex of the same species. In fact, due to the limited attention on female

aggression, it remains unclear whether the two sexes utilize the same neural circuitry for controlling aggression. Just like any comparative principle heavily used in evolutionary studies (Bullock, 1984; Miller et al., 2019), exploring the sex differences in aggression would ultimately reveal the core elements of the behavior and the underlying neural architecture, which is essential for the complete understanding of the neural control of aggression. Therefore, my thesis work aims to elucidate the circuit mechanisms underlying the sexually shared and dimorphic aggressive behaviors.

Sexual differences in *Drosophila* aggression

How the two sexes differ in aggression varies from one species to another. These sex differences can be roughly divided into qualitative and quantitative traits (Asahina, 2018). The former describes aggressive behaviors that are exclusively performed by one sex. The exclusivity is oftentimes the result of anatomical constraints. For example, only male elks develop weaponry antler. Thus, any aggressive expressions requiring the antler would be inevitably male-specific. The biting behavior of mice, on the other hand, is an example of the quantitative differences in aggression. Both male and female mice attack the opponent by biting, but male attacks last longer than the female ones (Unger et al., 2015). *Drosophila* is a unique and valuable model for studying the neural mechanisms underlying the sexual dimorphism in aggression because, even though male and female flies share similar body compositions, aggression performed by the two sexes differs significantly in both qualitative and quantitative ways (Asahina, 2018; Chan and Kravitz, 2007; Kravitz and Huber, 2003; Nilsen et al., 2004; Vrontou et al., 2006). It implies a specialized circuit organization that allows the implementations of both sexually shared and dimorphic aggressive behaviors in flies.

Sexual differences in fly aggression can be discussed from two different categories; motor actions and plasticity. Male and female flies display a variety of motor actions at each stage of aggressive encounters (Figure 1). During the appetitive phase, males and females display a similar set of actions but quantitative differences are seen in the transitions between these actions (Figure 1; Nilsen et al., 2004). When they detect the sensory cues emitted by a

conspecific target from a distance, both sexes orient and move toward the target, in an action described as 'approach' (Nilsen et al., 2004; Ueda and Kidokoro, 2002). Both sexes may touch the target with their forelegs to collect more sensory information or chase the target if the target is running away (Dankert et al., 2009; Nilsen et al., 2004). However, the behavioral transitions to and from 'approach' or 'chase' occur more frequently in males (Nilsen et al., 2004).

Regardless of sex, the interactions with the target may soon enter the consummatory phase after approaching or chasing, during which males and females display a mixture of sexually shared and dimorphic attacks (Figure 1). Males and females share some low-intensity actions (Nilsen et al., 2004), such as wing threat (threatening the opponent by lifting wings) or fencing (pushing the opponent with its legs). Some higher intensity actions are sex-specific, for example, lunge is mainly performed by males. They raise their upper body and slam on the opponent (Chen et al., 2002; Dankert et al., 2009; Hoyer et al., 2008; Nilsen et al., 2004). Headbutting, on the other hand, is a female-specific aggressive action. A female strikes the opponent with her head in a brief, forward movement (Figure 1; Chiu et al., 2021; Nilsen et al., 2004; Schretter et al., 2020). Fighting between males tends to escalate into tussling whereas female fights do not (Nilsen et al., 2004). Two observations are worth noting here. Firstly, 'lunging' was used to describe female aggression in early studies of female aggression (Nilsen et al., 2004; Ueda and Kidokoro, 2002). However, female lunging was not seen in our and other laboratory settings (Chiu et al., 2021; Vrontou et al., 2006). When applying the 'lunge' classifier to the female aggression videos, no lunging bouts were detected except for false positive predictions (Figure 2). The discrepancy may be due to genetic variations accumulated over time in the wild-type strain or differences in the behavioral setups. Nevertheless, we consider 'lunging' a male-specific action. Secondly, although it is not fully documented, there are qualitative differences in male versus female threats. Male threats are complex (Duistermars et al., 2018), which includes 45° or 90° wing lifting, charging, and pumping (180° bilateral wing extension). Female threats occur infrequently, and they are less noticeable. They flick their wings quickly rather than lift the wings to certain degrees like males do. In short, males and females share appetitive actions

during the beginning of an aggressive encounter, but they subsequently display sexually dimorphic actions, i.e., lunging or headbutting, to attack the opponent.

The word 'plasticity' is used loosely here to refer to quantitative changes in aggression that are caused by extrinsic or intrinsic factors (Table 1; Wei et al., 2021). These changes include increase or decrease in an individual's motivation or tendency to fight as well as shifts in the winning probability (Falkner et al., 2016; Penn et al., 2010; Stagkourakis et al., 2018; Yurkovic et al., 2006). Extrinsically, male and female aggression are both influenced by food source. Male aggressiveness is enhanced by the nutrient concentration of the food present during fighting whereas female aggressiveness is increased by the addition of yeast in the food during rearing period (Lim et al., 2014; Ueda and Kidokoro, 2002). The presence of females (mating partner) clearly promotes intermale aggression, however, it remains unclear if interfemale aggression is affected by the presence of males (Jung et al., 2020). Intrinsically, social isolation and mating experience are shown to enhance aggression in both males and females (Bath et al., 2017; Baxter et al., 2015; Ueda and Kidokoro, 2002; Wang et al., 2008). It is worth noting that, after mating, female aggressiveness is stimulated by the presence of sperm and sex peptide, which means that very different mechanisms are likely adopted to enhance male aggression after mating. Males establish hierarchical relationships during fighting, in other words, the winning male becomes more dominant than the loser (Chan and Kravitz, 2007; Nilsen et al., 2004; Simon and Heberlein, 2020; Vrontou et al., 2006). The experience of winning also significantly affects a male's performance in the subsequent battle (Penn et al., 2010; Trannoy et al., 2016; Yurkovic et al., 2006). Females, however, do not establish dominance during fighting (Nilsen et al., 2004; Vrontou et al., 2006). The behavioral plasticity implies reversable or permanent changes in the neural circuits that govern aggression. Before we can identify and study such changes, we have to first obtain a complete map of the aggression circuit in both sexes.

Neural circuits of aggression in *Drosophila*

Sex determination genes, *fruitless* (*fru*) and *doublesex* (*dsx*), are critical for establishing aggression circuits in flies (Chan and Kravitz, 2007; Koganezawa et al., 2016; Palavicino-

Maggio et al., 2019; Vrontou et al., 2006). The transcription of both genes undergoes alternative splicing, which gives rise to the sex-specific isoform expression in the two sexes (Demir and Dickson, 2005; Hoshijima et al., 1991; Nagoshi and Baker, 1990; Ryner et al., 1996). Importantly, the expression of the male-specific isoform of Fruitless (FruM) is required for specifying male-specific aggressive fighting patterns (Chan and Kravitz, 2007; Vrontou et al., 2006). Eliminating the expression of FruM in males not only reduces the overall level of aggressiveness, the mutant males also displayed female headbutting instead of lunges when attacking the opponent (Vrontou et al., 2006). Conversely, FruM-expressing females attack the opponent with lunges. A similar observation is made when the whole nervous system is feminized or masculinized by manipulating the expression of *transformer*, which affects the splicing of both *fru* and *dsx* (Chan and Kravitz, 2007). Together, it suggests that the expression of *fru* and *dsx* is required for constructing a functional aggression circuit especially in males.

Male and female nervous systems contain different numbers of *fru*- or *dsx*-expressing neurons: males have 1700-3300 *fru*-expression neurons and 400-700 *dsx*-expressing neurons whereas females have 3000 *fru*-expression neurons and 300-400 *dsx*-expressing neurons (Asahina, 2018). The number differences indicate the presence of sex-specific *fru*- or *dsx*-expressing neurons, which may play an important role in specifying sex-specific behaviors (Cachero et al., 2010; Nojima et al., 2021; Yu et al., 2010). It is important to note that the *fru* and *dsx* expression are not completely separate, which means that the synergistic effect of these two genes may be expected for defining the function of the overlapping populations (Asahina, 2018; Ishii et al., 2020; Wohl et al., 2020). Moreover, generating quantitative differences in neuronal composition is not the only way that these two genes contribute to sex-specific features of neural circuits. They also influence sex-specific connections by modifying the neuron morphology in both sexes. For example, FruM is required for the sexual differences in the dendritic location of the third-order olfactory neurons, aSP-f, which results in the male-specific wiring between DA1 projection neurons and aSP-f in males (Kohl et al., 2013). Such sex-specific wiring possibly contributes to the sex-specific responses to the same odor 11-*cis*-*vaccenyl* acetate (cVA; Kohl et al., 2013). It was shown recently that

the *dsx*-expressing anterior dorsal neurons (aDNs) are sexually dimorphic (Nojima et al., 2021). Such dimorphism is responsible for sex-specific sensory inputs to these neurons, which explains how aDNs are involved in different behaviors in males versus females (Nojima et al., 2021). Because *fru* and *dsx* are both transcription factors, it is also possible that they contribute to sex-specific function of the neurons by altering the gene profiling of the cells (Clough et al., 2014; Vernes, 2015). In short, the *fru* or *dsx* expression establishes the foundation for sex-specific features in neural circuits that govern sexually dimorphic behaviors including aggression (Asahina, 2018).

A group of *dsx*-expressing neurons labeled by NP2631^{dsxFLP}, called pC1 neurons, is shown to promote both male and female aggression (Koganezawa et al., 2016). Because the numbers of pC1 neurons differ a lot in males versus females, and because it has been shown that genetically identifiable pC1 subclasses are involved in distinct behaviors, it remains possible that male and female aggression are controlled by independent pC1 subclasses (Asahina, 2018; Deutsch et al., 2020; Koganezawa et al., 2016; Palavicino-Maggio et al., 2019; Schretter et al., 2020; Wang et al., 2020). This possibility is supported by the observations that a subset of FruM-expressing, male-specific pC1 neurons (P1a neurons), labeled by R71G01-Gal4^{FruFLP}, promotes male aggression, whereas a subclass of female-specific pC1 neurons, pC1d, promotes female aggression (Deutsch et al., 2020; Hoopfer et al., 2015; Schretter et al., 2020).

In addition to pC1 neurons, several groups of sex-specific neurons are shown to regulate male or female aggression. A subset of FruM, Tachykinin-expressing neurons and a subset of FruM, octopamine receptor-expressing neurons have been shown to promote male aggression (Asahina et al., 2014; Watanabe et al., 2017). These suggest that, on top of structural differences in circuits, sex-specific neural modulation may also contribute to the sexual dimorphism in fly aggression. Compared to males, female aggression is less studied. However, a subset of *fru*-expressing, female-specific aIPg neurons is recently identified to promote female aggression (Schretter et al., 2020). A critical question emerging from these studies of sex-specific neurons is whether the circuits that govern aggression in the two sexes

are completely independent. An argument against this hypothesis is that while some aggressive displays are shared by both sexes, it is less likely that males and females develop exclusive neural pathways to control similar motor actions. Along the same lines, aggression is critical for survival regardless of sex. It is thus reasonable to assume that some neural modules are shared by both sexes to regulate core features of aggression, such as aggressive arousal. Such shared modules have not been identified, which is the main motivation for my thesis work.

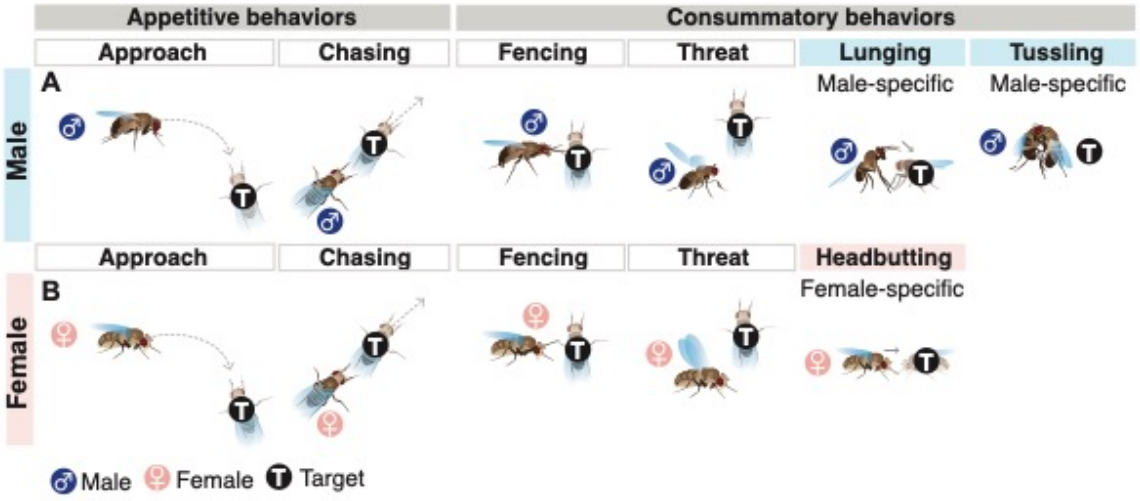


Figure 1. Sexually shared and dimorphic aggressive behaviors of *Drosophila*
(A) Male and (B) female aggressive behaviors. Behaviors performed during the appetitive phase are shared by both sexes whereas some of the behaviors displayed during consummatory phase are sex-specific. For example, lunging and tussling are observed only during intermale aggression. On the other hand, headbutting is mostly performed by females. Males normally only attack males. However, females attack both sexes.

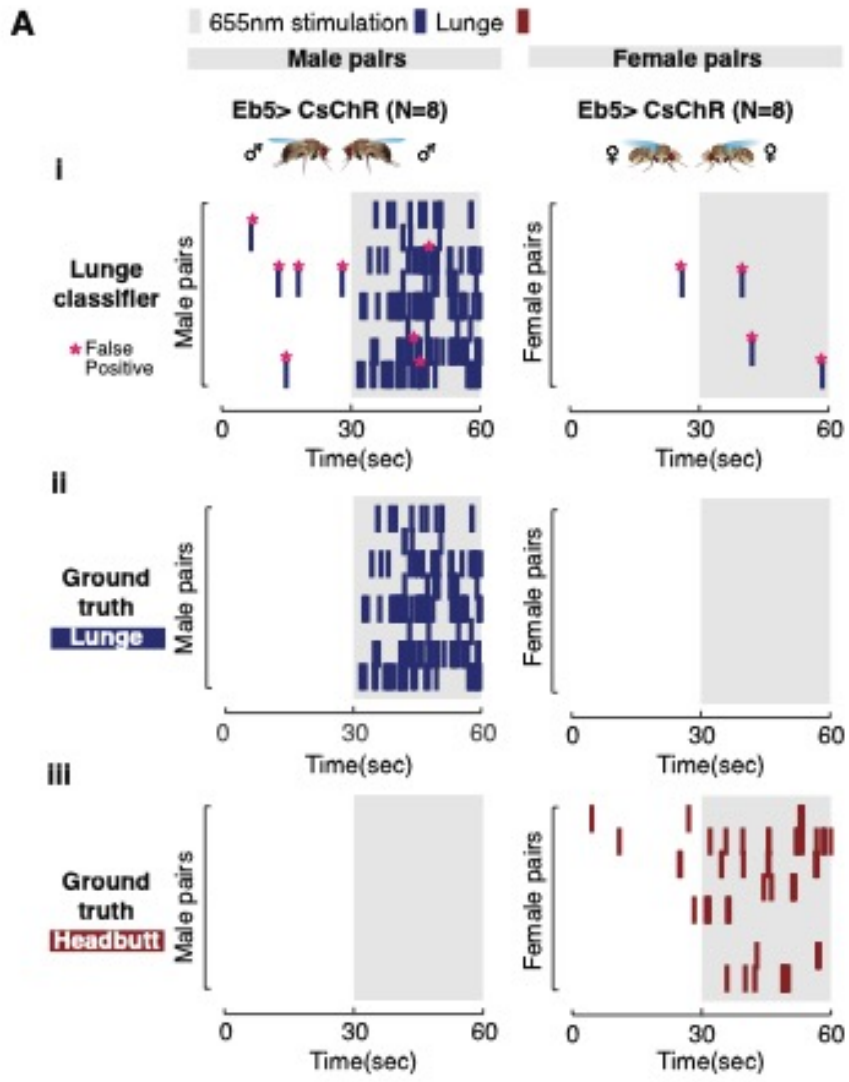


Figure 2. Lunging is not detected in female fights.
(A) Male and female aggression induced by activation of Eb5 neurons. (i) annotation predicted by the lunge classifier, and (ii-iii) the ground truth (manually curated annotations) for lunging and headbutting bouts. Grey box: 655nm photostimulation. Blue and red ticks: lunge and head-butt bouts, respectively. Red asterisks: false positives after validation against ground truth.

Behavioral plasticity					
	Extrinsic trigger		Intrinsic trigger		
	Food	Mating Partner	Social Isolation	Mating experience	Dominance
Male Aggression	↑	↑	↑	↑	Yes
Female Aggression	↑	ND	↑	↑	No

Table 1. Sexually dimorphic plasticity in fly aggression
Males and females differ in their responses to various extrinsic and intrinsic triggers for aggression. Upward arrows: increased aggressiveness. For mating experience, the arrow indicates that mated flies show a higher level of aggressiveness than virgins. ND: not determined. Yes/No: whether dominance is established between a pair of flies.

References

Asahina, K. (2018). Sex differences in *Drosophila* behavior: qualitative and quantitative dimorphism. Curr. Opin. Physiol. *6*, 35–45.

Asahina, K., Watanabe, K., Duistermars, B.J., Hoopfer, E., González, C.R., Eyjólfsdóttir, E.A., Perona, P., and Anderson, D.J. (2014). Tachykinin-expressing neurons control male-specific aggressive arousal in *Drosophila*. Cell *156*, 221–235.

Bath, E., Bowden, S., Peters, C., Reddy, A., Tobias, J.A., Easton-Calabria, E., Seddon, N., Goodwin, S.F., and Wigby, S. (2017). Sperm and sex peptide stimulate aggression in female *Drosophila*. Nat. Ecol. Evol. *1*, 0154.

Baxter, C.M., Barnett, R., and Dukas, R. (2015). Aggression, mate guarding and fitness in male fruit flies. Anim. Behav. *109*, 235–241.

Bullock, T.H. (1984). Comparative neuroscience holds promise for quiet revolutions. Science *225*, 473–478.

Cachero, S., Ostrovsky, A.D., Yu, J.Y., Dickson, B.J., and Jefferis, G.S.X.E. (2010). Sexual dimorphism in the fly brain. Curr. Biol. *20*, 1589–1601.

Chan, Y.-B., and Kravitz, E.A. (2007). Specific subgroups of FruM neurons control sexually dimorphic patterns of aggression in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. *104*, 19577–19582.

Chen, S., Lee, A.Y., Bowens, N.M., Huber, R., and Kravitz, E.A. (2002). Fighting fruit flies: A model system for the study of aggression. Proc. Natl. Acad. Sci. *99*, 5664–5668.

Chiu, H., Hoopfer, E.D., Coughlan, M.L., Pavlou, H.J., Goodwin, S.F., and Anderson, D.J. (2021). A circuit logic for sexually shared and dimorphic aggressive behaviors in *Drosophila*. Cell *184*, 507-520.e16.

Clough, E., Jimenez, E., Kim, Y.-A., Whitworth, C., Neville, M.C., Hempel, L.U., Pavlou, H.J., Chen, Z.-X., Sturgill, D., Dale, R.K., et al. (2014). Sex- and tissue-specific functions of *Drosophila doublesex* transcription factor target genes. Dev. Cell *31*, 761–773.

Dankert, H., Wang, L., Hoopfer, E.D., Anderson, D.J., and Perona, P. (2009). Automated monitoring and analysis of social behavior in *Drosophila*. Nat. Methods *6*, 297–303.

Demir, E., and Dickson, B.J. (2005). *fruitless* splicing specifies male courtship behavior in *Drosophila*. Cell *121*, 785–794.

Deutsch, D., Pacheco, D., Encarnacion-Rivera, L., Pereira, T., Fathy, R., Clemens, J., Girardin, C., Calhoun, A., Ireland, E., Burke, A., et al. (2020). The neural basis for a persistent internal state in *Drosophila* females. ELife *9*, e59502.

Duistermars, B.J., Pfeiffer, B.D., Hoopfer, E.D., and Anderson, D.J. (2018). A brain module for scalable control of complex, multi-motor threat displays. Neuron *100*, 1474-1490.e4.

Falkner, A.L., Grosenick, L., Davidson, T.J., Deisseroth, K., and Lin, D. (2016). Hypothalamic control of male aggression-seeking behavior. Nat. Neurosci. *19*, 596–604.

Hashikawa, K., Hashikawa, Y., Lischinsky, J., and Lin, D. (2018). The neural mechanisms of sexually dimorphic aggressive behaviors. Trends Genet. *34*, 755–776.

Hashikawa, Y., Hashikawa, K., Falkner, A.L., and Lin, D. (2017). Ventromedial hypothalamus and the generation of aggression. Front. Syst. Neurosci. *11*, 94.

Hoopfer, E.D. (2016). Neural control of aggression in *Drosophila*. Curr. Opin. Neurobiol. *38*, 109–118.

Hoopfer, E.D., Jung, Y., Inagaki, H.K., Rubin, G.M., and Anderson, D.J. (2015). P1 interneurons promote a persistent internal state that enhances inter-male aggression in *Drosophila*. ELife *4*, e11346.

Hoshijima, K., Inoue, K., Higuchi, I., Sakamoto, H., and Shimura, Y. (1991). Control of *doublesex* alternative splicing by transformer and transformer-2 in *Drosophila*. Science *252*, 833–836.

Hoyer, S.C., Eckart, A., Herrel, A., Zars, T., Fischer, S.A., Hardie, S.L., and Heisenberg, M. (2008). Octopamine in male aggression of *Drosophila*. Curr. Biol. *18*, 159–167.

Ishii, K., Wohl, M., DeSouza, A., and Asahina, K. (2020). Sex-determining genes distinctly regulate courtship capability and target preference via sexually dimorphic neurons. ELife *9*, e52701.

Jung, Y., Kennedy, A., Chiu, H., Mohammad, F., Claridge-Chang, A., and Anderson, D.J. (2020). Neurons that function within an integrator to promote a persistent behavioral state in *Drosophila*. Neuron *105*, 322-333.e5.

Koganezawa, M., Kimura, K., and Yamamoto, D. (2016). The neural circuitry that functions as a switch for courtship versus aggression in *Drosophila* males. Curr. Biol. *26*, 1395–1403.

Kohl, J., Ostrovsky, A.D., Frechter, S., and Jefferis, G.S.X.E. (2013). A bidirectional circuit switch reroutes pheromone signals in male and female brains. Cell *155*, 1610–1623.

Kohn, S.R., and Asnis, G.M. Aggression in psychiatric disorders. In Neurobiology of Aggression, (Humana Press, Totowa, NJ), pp. 135–149.

Kravitz, E.A., and Huber, R. (2003). Aggression in invertebrates. Curr. Opin. Neurobiol. *13*, 736–743.

Lane, S.D., Kjome, K.L., and Moeller, F.G. (2011). Neuropsychiatry of aggression. Neurol. Clin. *29*, 49–64.

Laredo, S.A., Villalon Landeros, R., and Trainor, B.C. (2014). Rapid effects of estrogens on behavior: Environmental modulation and molecular mechanisms. Front. Neuroendocrinol. *35*, 447–458.

Lee, H., Kim, D.-W., Remedios, R., Anthony, T.E., Chang, A., Madisen, L., Zeng, H., and Anderson, D.J. (2014). Scalable control of mounting and attack by Esr1+ neurons in the ventromedial hypothalamus. Nature *509*, 627–632.

Lim, R.S., Eyjólfsdóttir, E., Shin, E., Perona, P., and Anderson, D.J. (2014). How food controls aggression in *Drosophila*. PLoS ONE *9*, e105626.

Miller, C.T., Hale, M.E., Okano, H., Okabe, S., and Mitra, P. (2019). Comparative principles for next-generation neuroscience. Front. Behav. Neurosci. *13*, 12.

Nagoshi, R.N., and Baker, B.S. (1990). Regulation of sex-specific RNA splicing at the *Drosophila doublesex* gene: cis-acting mutations in exon sequences alter sex-specific RNA splicing patterns. Genes Dev. *4*, 89–97.

Nilsen, S.P., Chan, Y.-B., Huber, R., and Kravitz, E.A. (2004). Gender-selective patterns of aggressive behavior in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. *101*, 12342–12347.

Nojima, T., Rings, A., Allen, A.M., Otto, N., Verschut, T.A., Billeter, J.-C., Neville, M.C., and Goodwin, S.F. (2021). A sex-specific switch between visual and olfactory inputs underlies adaptive sex differences in behavior. Curr. Biol. *31*, 1175-1191.e6.

Palavicino-Maggio, C.B., Chan, Y.-B., McKellar, C., and Kravitz, E.A. (2019). A small number of cholinergic neurons mediate hyperaggression in female *Drosophila*. Proc. Natl. Acad. Sci. *116*, 17029–17038.

Penn, J.K.M., Zito, M.F., and Kravitz, E.A. (2010). A single social defeat reduces aggression in a highly aggressive strain of *Drosophila*. Proc. Natl. Acad. Sci. *107*, 12682–12686.

Ryner, L.C., Goodwin, S.F., Castrillon, D.H., Anand, A., Villella, A., Baker, B.S., Hall, J.C., Taylor, B.J., and Wasserman, S.A. (1996). Control of male sexual behavior and sexual orientation in *Drosophila* by the *fruitless* gene. Cell *87*, 1079–1089.

Schretter, C.E., Aso, Y., Robie, A.A., Dreher, M., Dolan, M.-J., Chen, N., Ito, M., Yang, T., Parekh, R., Branson, K.M., et al. (2020). Cell types and neuronal circuitry underlying female aggression in *Drosophila*. ELife *9*, e58942.

Siegel, A., and Skog, D. (1970). Effects of electrical stimulation of the septum upon attack behavior elicited from the hypothalamus in the cat. Brain Res. *23*, 371–380.

Simon, J.C., and Heberlein, U. (2020). Social hierarchy is established and maintained with distinct acts of aggression in male *Drosophila melanogaster*. J. Exp. Biol. *223*, jeb232439.

Stagkourakis, S., Spigolon, G., Williams, P., Protzmann, J., Fisone, G., and Broberger, C. (2018). A neural network for intermale aggression to establish social hierarchy. Nat. Neurosci. *21*, 834–842.

Takahashi, A., Chung, J.-R., Zhang, S., Zhang, H., Grossman, Y., Aleyasin, H., Flanigan, M.E., Pfau, M.L., Menard, C., Dumitriu, D., et al. (2017). Establishment of a repeated social defeat stress model in female mice. Sci. Rep. *7*, 12838.

Tieger, T. (1980). On the biological basis of sex differences in aggression. Child Dev. *51*, 943–963.

Trannoy, S., Penn, J., Lucey, K., Popovic, D., and Kravitz, E.A. (2016). Short and long-lasting behavioral consequences of agonistic encounters between male *Drosophila melanogaster*. Proc. Natl. Acad. Sci. *113*, 4818–4823.

Ueda, A., and Kidokoro, Y. (2002). Aggressive behaviours of female *Drosophila melanogaster* are influenced by their social experience and food resources. Physiol. Entomol. *27*, 21–28.

Unger, E.K., Burke, K.J., Yang, C.F., Bender, K.J., Fuller, P.M., and Shah, N.M. (2015). Medial amygdalar aromatase neurons regulate aggression in both sexes. Cell Rep. *10*, 453–462.

Vernes, S.C. (2015). Genome wide identification of Fruitless targets suggests a role in upregulating genes important for neural circuit formation. Sci. Rep. *4*, 4412.

Vrontou, E., Nilsen, S.P., Demir, E., Kravitz, E.A., and Dickson, B.J. (2006). *fruitless* regulates aggression and dominance in *Drosophila*. Nat. Neurosci. *9*, 1469–1471.

Wang, F., Wang, K., Forknall, N., Patrick, C., Yang, T., Parekh, R., Bock, D., and Dickson, B.J. (2020). Neural circuitry linking mating and egg laying in *Drosophila* females. Nature *579*, 101–105.

Wang, L., Dankert, H., Perona, P., and Anderson, D.J. (2008). A common genetic target for environmental and heritable influences on aggressiveness in *Drosophila*. Proc. Natl. Acad. Sci. *105*, 5657–5663.

Watanabe, K., Chiu, H., Pfeiffer, B.D., Wong, A.M., Hoopfer, E.D., Rubin, G.M., and Anderson, D.J. (2017). A circuit node that integrates convergent input from neuromodulatory and social behavior-promoting neurons to control aggression in *Drosophila*. Neuron *95*, 1112-1128.e7.

Wei, D., Talwar, V., and Lin, D. (2021). Neural circuits of social behaviors: innate yet flexible. Neuron (In press). https://doi.org/10.1016/j.neuron.2021.02.012.

Wohl, M., Ishii, K., and Asahina, K. (2020). Layered roles of *fruitless* isoforms in specification and function of male aggression-promoting neurons in *Drosophila*. ELife *9*, e52702.

Wong, L.C., Wang, L., D'Amour, J.A., Yumita, T., Chen, G., Yamaguchi, T., Chang, B.C., Bernstein, H., You, X., Feng, J.E., et al. (2016). Effective modulation of male aggression through lateral septum to medial hypothalamus projection. Curr. Biol. *26*, 593–604.

Yu, J.Y., Kanai, M.I., Demir, E., Jefferis, G.S.X.E., and Dickson, B.J. (2010). Cellular organization of the neural circuit that drives *Drosophila* courtship behavior. Curr. Biol. *20*, 1602–1614.

Yurkovic, A., Wang, O., Basu, A.C., and Kravitz, E.A. (2006). Learning and memory associated with aggression in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. *103*, 17519–17524.

Zwarts, L., Versteven, M., and Callaerts, P. (2012). Genetics and neurobiology of aggression in *Drosophila*. Fly (Austin) *6*, 35–48.

.

C h a p t e r 2

A CIRCUIT LOGIC FOR SEXUALLY SHARED AND DIMORPHIC AGGRESSIVE BEHAVIORS IN *DROSOPHILA*

Chiu, H., Hoopfer, E.D., Coughlan, M.L., Pavlou, H.J., Goodwin, S.F., and Anderson, D.J. (2021). "A circuit logic for sexually shared and dimorphic aggressive behaviors in *Drosophila*". Cell *184*, pp. 507-520. doi: 10.1016/j.cell.2020.11.048

Summary

Aggression involves both sexually monomorphic and dimorphic actions. How the brain implements these two types of actions is poorly understood. We have identified three cell types that regulate aggression in *Drosophila*: one type is sexually shared, and the other two are sex-specific. We show that shared CAP neurons mediate aggressive approach in both sexes, whereas functionally downstream dimorphic but homologous cell types, called MAP in males and fpC1 in females, control dimorphic attack. These symmetric circuits underlie the divergence of male and female aggressive behaviors, from their monomorphic appetitive/motivational to their dimorphic consummatory phases. The strength of the monomorphic→dimorphic functional connection is increased by social isolation in both sexes, suggesting that it may be a locus for isolation-dependent enhancement of aggression. Together, these findings reveal a circuit logic for the neural control of behaviors that include both sexually monomorphic and dimorphic actions, which may generalize to other organisms.

Introduction

Males and females of a given species often show sex-specific differences in behavior (reviewed in Dulac and Kimchi, 2007; Manoli et al., 2013). These dimorphic behaviors can

be roughly divided into two categories: "pure" dimorphic behaviors, in which males and females exhibit non-overlapping motor patterns to achieve a similar goal; and "mixed" monomorphic-dimorphic behaviors, in which certain actions are common to both sexes, while other actions are dimorphic. Extensive research in multiple species has shown that "pure" sexually dimorphic behaviors (e.g., mating) are controlled by sexually dimorphic brain circuits (reviewed in Asahina, 2018; Yamamoto and Koganezawa, 2013; Yang and Shah, 2014). However, much less is known about the configuration of circuits that control and coordinate "mixed" behaviors.

Aggressive behavior in *Drosophila* exhibits a mixed pattern: a monomorphic appetitive and a dimorphic consummatory phase (Chan and Kravitz, 2007; Chen et al., 2002; Craig, 1917; Hoyer et al., 2008; Lorenz, 1950; Nilsen et al., 2004; Vrontou et al., 2006). Both sexes approach the opponent during the appetitive phase and then initiate the consummatory attack: males lunge and tussle while females headbutt (Chan and Kravitz, 2007; Nilsen et al., 2004; Vrontou et al., 2006). A great deal has been learned about neural circuit nodes that control aggression in *Drosophila* males (reviewed in Hoopfer, 2016; Kravitz and Fernández, 2015). Less work has been done on female aggression (Deutsch et al., 2020; Palavicino-maggio et al., 2019; Schretter et al., 2020) and even less on the control of the monomorphic vs. dimorphic aspects of this behavior.

Two extreme models could explain the control of this mixed mono-/dimorphic behavior by the brain. In one model, all phases of male and female aggression are controlled by sex-specific circuit nodes. In support of this, most identified neurons controlling male and female aggression in flies are sex-specific, for example, Tk^{FruM}, aSP2, and P1a cells in males, as well as aIP-g and pC1d neurons in females (Asahina et al., 2014; Deutsch et al., 2020; Hoopfer et al., 2015; Palavicino-maggio et al., 2019; Schretter et al., 2020; Watanabe et al., 2017). Furthermore, manipulation of sex-determination genes like *fruitless* or *transformer* switches the pattern of sex-specific fighting (Chan and Kravitz, 2007; Vrontou et al., 2006). In an alternative model, the mono- and dimorphic phases of aggression could be controlled by mono- and dimorphic circuit nodes, respectively. The fact that male and female aggression

can be induced by common external or internal triggers (Lim et al., 2014; Ueda and Kidokoro, 2002; Wang et al., 2008) is consistent with such a model. However, sexually shared neurons that control monomorphic features of aggression have not yet been identified.

Here we identify a pair of sexually shared neurons whose activation increases aggressive approach towards same-sex targets in both males and females. We also identify homologous, male- and female-specific interneurons that promote male- or female-specific attack behavior, respectively. We show that the dimorphic neurons are functionally downstream of the common neurons in both sexes. Moreover, we demonstrate that the functional connectivity of this circuit motif is strengthened in isolated males and females, which are more aggressive than their group-housed counterparts (Ueda and Kidokoro, 2002; Wang et al., 2008). Together, these data suggest a circuit logic for the neural control of a "mixed" monomorphic-dimorphic social behavior, and identify a potential locus for experience-dependent modulation of this behavior.

Results

Fly aggression consists of sexually monomorphic and dimorphic behaviors

We first characterized aggression in wild-type flies of both sexes and developed automated classifiers for quantifying the appetitive (approach) and the consummatory (attack) phases of aggression (Figure 1; Craig, 1917; Tinbergen, 1951). An approach bout occurs similarly in both sexes, and typically involves three motor elements: orientation from a distance, advance, and contact (Figures 1A and S1A; Video S1). In contrast, attack is sexually dimorphic: in males it includes lunging, in which the behaving fly raises its upper body with its front legs up, and slams down onto its target (Chen et al., 2002; Hoyer et al., 2008); in females it includes headbutting which comprises a quick horizontal thrust in the direction of the target fly, resulting in contact (Figure 1A; Video S2; Nilsen et al., 2004). These

monomorphic (approach) and dimorphic (lunge/headbutt) aggressive behaviors could be reliably detected by our behavioral classifiers (Figure S1D; Table S2).

Aggression can be promoted by social isolation or by food in both males and females (Lim et al., 2014; Ueda and Kidokoro, 2002; Wang et al., 2008). However, direct, quantitative comparisons of these effects on the two sexes have not been performed. We therefore investigated how males and females respond to food or social isolation (SI), during or prior to aggressive encounters, respectively. Both SI and food increased aggression in both sexes, albeit to different extents (Figures 1B and 1C). However, the group-housed females showed a higher level of baseline aggression than their male counterparts (Figures 1Bi and 1Ci, GH). Thus, male and female aggressiveness can be increased by similar environmental influences, despite their dimorphic attack behavior. These influences may act in parallel on sexually dimorphic circuits (Figure 1D, Model 1), or via a module common to both sexes (Figure 1D, Model 2). To distinguish between these models, we next investigated the relationship between neural circuits controlling aggression in males versus females.

Identification of sexually shared and dimorphic aggression-promoting neurons

A distinguishing feature of Model 2 is an aggression-promoting node common to both sexes (Figure 1D, Model 2, 'C'). We therefore searched for such neurons, by re-screening in females Gal4 drivers identified previously in a large-scale screen for aggression-promoting neurons in males (Hoopfer et al., 2015). This screen yielded a promising candidate, line R60G08-Gal4 (R60G08 neurons, henceforth), optogenetic activation of which strongly promoted both approach, and dimorphic attack, in both sexes (Figure S2A).

R60G08-Gal4 drives expression in roughly 80 neurons in males and 64 neurons in females (Figure S2B). This sex difference could reflect quantitative or qualitative differences in aggression neurons. We first confirmed that aggression is promoted by the R60G08 neurons in the brain but not in the ventral nerve cord of both sexes (Figure S2C). To narrow down the subset of neurons that control aggression, we used an "enhancer bashing" strategy to further fractionate the R60G08 population (Figure 2A) (Hobert and Kratsios, 2019; Luo et

al., 2008). To this end, we divided the 1.5-kb R60G08 *cis*-regulatory module (CRM) sequence (Jenett et al., 2012; Pfeiffer et al., 2008) into five 0.5-kb partially overlapping fragments and generated corresponding Gal4 drivers inserted into the same genomic locus (Enhancer bashing (Eb) 1-5 Gal4s).

This approach yielded new Gal4 drivers with sparse labeling of R60G08 neuron subsets in the two sexes (Figures 2B and S3A). Among these, optogenetic activation of Eb5-Gal4 neurons (Eb5 neurons, henceforth) triggered robust male and female aggression (Figures 2C), suggesting that these cells may account for R60G08 neuron-induced aggression. To test this hypothesis, we activated R60G08 neurons in the parental Gal4 driver line, while "subtracting" Eb5 neurons using Gal80 (R60G08+/Eb5-). Such activation yielded little or no detectable increase in male or female aggression (Figure S3B). These data indicate that Eb5 neurons are required for R60G08 neuron-induced aggression.

Eb5-Gal4 labeled a pair of neurons in each male hemi-brain and a single neuron in each female hemi-brain (Figure 2B). A subset of these neurons expressed *dsx-Gal4,* but none expressed FruM (Figure S3C). To further subdivide the two Eb5 neurons identified in males, we visualized their morphology using photo-activatable GFP (PA-GFP) (Datta et al., 2008; Ruta et al., 2010). This revealed two morphologically distinct cell types. One of the cell types resembled the Eb5 neuron labeled in females, whereas the other was dissimilar and appeared only in males (Figures 2Di-ii and 2Ei). We then generated intersectional drivers that separately labeled these two classes of Eb5 neurons in males, using R22F05 CRM (Figures 2Diii-v, 2Eiii, S3D, S3E, and S4Ai; Hoopfer et al., 2015). We refer to these cells as CAP (Common Aggression-Promoting neurons) and MAP neurons (Male-specific Aggression-Promoting neurons), respectively. Both the CAP and MAP drivers labeled neurons exclusively in the brain (Figure S4B).

CAP neurons promote the approach phase of aggression in both sexes

We first investigated the function of the shared CAP neurons in males and females. We optogenetically activated CAP neurons and examined the effect on interactions between pairs

of freely moving group-housed (GH) flies of both sexes. When the CAP neurons were activated, both males and females initiated significantly more approach bouts during the stimulation period (Figure 3Ai).

We next asked whether CAP neurons evoke generic approach towards any object, and whether this approach behavior was biased towards same- or opposite-sex flies. To this end, we developed an approach preference assay, in which we provided pairs of different "target objects" in the chamber, and asked whether the GH tester fly preferentially approached one of the targets during CAP activation. Initially, we gave each tester fly a choice between a same-sex dead fly or a fly-sized object (magnet; Figure 3Bi). Both sexes of tester flies exhibited a higher percentage of approach bouts directed toward the fly target (Figure 3Bi). This result suggests that stimulation of CAP neurons preferentially promotes approach to conspecifics, relative to an inanimate object.

We then investigated whether the approach promoted by CAP neurons was biased towards same- or opposite-sex conspecifics. *Drosophila* males normally do not attack females (even if aggression-promoting neurons are activated (Figure S5A)), and female flies of other species most frequently attack females, in competition for oviposition sites (Fernández et al., 2010; Shelly, 1999). Consistent with this, during CAP stimulation male testers preferred male over female targets, whereas female testers showed the opposite tendency (Figure 3Bii; Video S3). The tester's target preference was not affected by the duration of its interaction with the targets prior to CAP stimulation (Figure S5B). More importantly, if both targets were female, no difference in target preference was observed between CAP-stimulated male vs. female testers (Figure 3Biii). Furthermore, wing extension, a male courtship behavior (Bennet-Clark and Ewing, 1967; von Philipsborn et al., 2011) that was induced naturally by the presence of a female (alive or dead), was not promoted by CAP activation (Figure 3Aiii). These data therefore indicate that CAP neurons promote approach towards sex-appropriate targets of aggressive behavior.

Lastly, we examined how aggression was affected in the two sexes when the CAP neurons were silenced. To do this, we enhanced natural aggressiveness using social isolation, and

silenced the CAP neurons using the inwardly-rectifying potassium channel Kir2.1 (Baines et al., 2001). Silencing the CAP neurons greatly decreased overall aggressiveness in both sexes, as indicated by a significant reduction in the number of both approach and attack bouts (Figure 3C). The locomotor activity of CAP>Kir2.1 flies was comparable to that of CAP>GFP controls (Figure S5C), suggesting that the effect to inhibit aggression is not due to a general reduction in vigor. Conditional silencing with the optogenetic inhibitor, GtACR (Mohammad et al., 2017) or the temperature-dependent inhibitory effector, Shibire^{ts}, was not feasible due to adverse effects of green light or the non-permissive temperature on aggression in control flies. Together, these data indicate that although activation of CAP neurons only promotes approach, the activity of these cells is required for both the approach and attack phases of male and female aggression. This further supports the idea that CAP neurons promote aggressive rather than generic approach.

CAP neurons exhibit sex differences in aggression-promoting thresholds

As part of our optogenetic protocol, we performed a stimulation titration experiment, in which we activated the CAP neurons in males and females with five different light intensities, titrated from 0.1 to 0.62 μW/mm^2 (Figure 4A). Unexpectedly, this experiment revealed sex differences in the threshold for CAP stimulation-induced aggression. In GH females, approach was elicited at low photostimulation intensities (0.1 μW/mm^2), while headbutting was elicited at higher intensities (0.43 μW/mm^2; Figures 4Ai ① and 4Aiii ③). By contrast, in GH males the lunging behavior was not evoked, even at the highest light stimulation intensity tested (Figure 4Aiv). Furthermore, in females the threshold intensity for eliciting approach (0.1 μW/mm^2; Figure 4Ai ①) was substantially (4-fold) lower than in males (0.43 μW/mm^2; Figure 4Aii ③).

We asked what neural mechanism(s) might underlie the sex differences in the effects of CAP stimulation. We hypothesized that in females, CAP neurons might be intrinsically more excitable than in males. To test this idea, we performed an *in vivo* all-optical stimulation and imaging experiment to quantify the responses of CAP neurons to optogenetic activation in

each sex (Figure 4Bi). When activated using photostimulation at the same frequency and intensity, female CAP neurons showed a slightly higher GCaMP fluorescence increase than did male CAP neurons (Figure 4Bi; p=0.034, but non-significant after the Bonferroni correction for multiple comparisons). Importantly, the fluorescence intensities of both Chrimson::tdTomato and GCaMP in the CAP neurons were statistically indistinguishable between sexes (Figure 4Bii). While this sex difference in the excitability is consistent with the observed difference in the threshold for CAP-evoked aggression, other mechanisms are likely involved.

Together, our titration experiments indicated that in GH females, CAP neurons can evoke approach and attack (headbutting), in a scalable manner. In contrast, CAP neurons only evoked approach behavior in GH males. One explanation for this difference is that in males, CAP neurons do not connect with neurons that control attack. Alternatively, they may make such a connection, but one that is weaker than in females. To distinguish these alternatives, we next sought to identify attack-promoting neurons in males and to determine their functional interaction with CAP neurons.

MAP neurons control the attack phase of aggression in males and are functionally downstream of CAP neurons

First, we asked whether MAP neurons might control the attack phase of aggression in males. Indeed, in GH males, optogenetic activation of MAP neurons strongly promoted lunging, while activation of CAP neurons was insufficient to do so (Figure 5A). Furthermore, in contrast to CAP neurons, activating MAP neurons did not increase the number of approach bouts initiated during photostimulation, relative to baseline or to genetic controls (Figures 3Aii and S4Aii). Consequently, MAP-induced lunging typically occurred during serendipitous close encounters between flies (Video S1). Consistent with these observations, the frequency of lunge bouts, but not that of approach bouts, was significantly diminished when MAP neurons were silenced using Kir2.1 (Figures 5B and S5C). To further confirm these results, we activated both CAP and MAP neurons simultaneously using Chrimson, while specifically inhibiting MAP neurons with Kir2.1 (Figure 5C). Both approach and

lunging behaviors were strongly increased when CAP and MAP neurons were co-activated (Figure 2Ci-ii). The number of lunge bouts and the fraction of approaches leading to lunges were suppressed by MAP inhibition, but not the number of approaches initiated (Figure 5C, cf. Figure 3C). Taken together, these results suggest that the sexually shared CAP neurons are required for the initiation of approach in both males and females, while the male-specific MAP neurons are required for male-specific attack (lunging) (Figure 5E); both populations may contribute to the transition from approach to attack.

Anatomical analysis showed that CAP and MAP neurites lie in close proximity (Figure 2Bi-ii), suggesting that MAP neurons might be a downstream target of CAP neurons in males. To test this hypothesis, we performed *in vivo* calcium imaging in MAP neurons while optogenetically stimulating CAP neurons. Indeed, GCaMP fluorescence signals in MAP neurons were significantly elevated during CAP photostimulation, suggesting that the former lie functionally downstream of the latter (Figure 6Aii). However, these experiments do not distinguish whether this functional connection is direct (monosynaptic) or indirect.

fpC1 neurons represent a functional homolog of MAP neurons in females

The foregoing results indicated that in GH males, CAP activation promotes approach, and MAP activation in turn promotes attack. Paradoxically, in GH females, strong CAP activation could evoke both approach and attack (Figures 4Aiii and 5D), but no MAP neurons were labeled by the Eb5 or MAP drivers. One explanation for this paradox is that CAP neurons in females directly control both approach and attack. Alternatively, females may contain a sex-specific homolog of MAP neurons, which was not labeled by our Gal4 drivers, and which controls headbutting (attack). We therefore searched for MAP-like neurons in females, using anatomical methods.

The morphology of the MAP cells resembles that of pC1 neurons, a cluster of cells labeled by the intersection of NP2631-Gal4 and *dsx*-Flp (Figure 2E). Thermogenetic activation of the $pC1^{NP2631;dsxFlp}$ cluster was reported to promote aggression in both males and females (Ishii et al., 2020; Koganezawa et al., 2016). NBlast (Costa et al., 2016) searches performed

using MAP neurite traces as a query returned several pC1 clusters among the top hits (Figure S4C). However, MAP neurons were not labeled by NP2631-Gal4 (Figure S4D), suggesting they might be a distinct pC1 subtype. The numbers of pC1 neurons differ in the two sexes (53-65 and 5-10 neurons per hemisphere in males and females, respectively; reviewed in Asahina, 2018), and these neurons can be further divided into morphologically distinct subtypes (Costa et al., 2016; Wang et al., 2020). This raised the possibility that a functional homolog of MAP neurons might exist among pC1 neurons in females.

Recently, a group of female-specific neurons intersectionally labeled by R26E01-Gal4 and *dsx*-Flp were shown to trigger intense female aggression when thermogenetically activated (Palavicino-maggio et al., 2019). These cells were identified as a female-specific subtype of pC1 neurons. We therefore labeled similar cells using a different intersectional strategy, in which R26E01-AD and dsx-DBD hemi-drivers were used to generate a split-Gal4 (Figure 6Bi). Cells labeled by this split-Gal4 driver in females exhibited a main projection pattern similar to that of MAP neurons, but additionally extended a short, lateral branch and a long, ventral branch (cf. Figure 6Ai vs. 6Bi). No neurons were labeled in the male brain (Figure S4Ei). The overall morphology of these cells resembles that of pC1d neurons, a subtype of pC1 neurons that recently has been shown to promote female aggression (Deutsch et al., 2020; Schretter et al., 2020; Wang et al., 2020). We therefore refer to these neurons as "fpC1" cells. Unfortunately, it was not possible to establish definitively correspondence between fpC1 and pC1d neurons, due to driver incompatibility. Because of their anatomic similarity to MAP neurons in males, these data raised the possibility that fpC1 neurons might be female analogs or homologs of MAP neurons.

Because previous studies of R26E01-Gal4; dsx-FLP neurons used thermogenetic activation, and did not include analysis of approach behavior (Palavicino-maggio et al., 2019), we first activated these neurons optogenetically (Figure 6C). Such activation promoted female attack (headbutting; Figure 6Cii), but not approach behavior (Figure 6Ci). We confirmed that this phenotype is due to activation of fpC1 neurons in the brain, but not of those in the ventral nerve cord (Figure S4Eii). Finally, we silenced fpC1 neurons using Kir2.1. This

manipulation strongly suppressed headbutting, but did not affect approach (Figures 6D and S5C). Thus, the behavioral phenotype of activating and silencing fpC1 neurons in females was analogous to that of activating and silencing MAP neurons in males (Figures 3Aii, 5Aii, and 5B).

Since MAP neurons receive excitatory input from CAP neurons in males, we hypothesized that fpC1 neurons may likewise receive excitatory input from CAP neurons in females. Indeed, fpC1 neurons responded strongly to CAP activation (Figure 6Bii), suggesting that they are indeed direct or indirect synaptic targets of the latter cells. Next, we performed a behavioral epistasis experiment, in which CAP neurons were strongly activated while silencing fpC1 neurons with Kir2.1 (Figure 6E). In this compound genotype, CAP-induced female aggression was suppressed. Together, these data suggest that fpC1 neurons are functionally as well as physiologically downstream of CAP cells in females. If so, it would suggest an analogous circuit motif controlling aggression in the two sexes, in which the sexually shared CAP neurons target MAP neurons in males, and fpC1 neurons in females (Figure 6F).

We observed that CAP stimulation caused a relatively greater increase in $\Delta F/F$ in fpC1 than in MAP neurons (Figures 6Aii vs. 6Bii, Stim). This difference was not due to sex differences in Chrimson expression in CAP neurons (Figure 4Bii), or in baseline GCaMP expression in MAP vs. fpC1 neurons (Figure S4F). One possibility is that the CAP→fpC1 functional connection in females is stronger than the CAP→MAP connection in males. This would be consistent with our observation that strong CAP activation in GH females evoked both approach and attack, while in GH males it evoked only approach. Alternatively, fpC1 neurons may be intrinsically more excitable than MAP neurons.

Social isolation enhances aggressiveness by strengthening circuit connectivity

The effect of social isolation to increase aggression has been observed in many species (Chiara et al., 2019; Toth et al., 2008; Ueda and Kidokoro, 2002; Wang et al., 2008; Zelikowsky et al., 2018). We thus wondered how social isolation can boost aggressiveness

in both males and females, despite their differences in aggression circuitry and behavior. Social isolation may act to increase the excitability of CAP neuron in both sexes, the excitability of both MAP and fpC1 neurons, or the strength of the connection between CAP→MAP/fpC1 neurons. To address this, we investigated the excitability and functional connectivity of these neurons in group- (GH) vs single-housed (SH) flies.

We first compared the excitability of each of the three neuron types in GH vs. SH flies, by co-expressing Chrimson and GCaMP in each cell, and optogenetically activating and imaging them. In both sexes, the response amplitude of each of these neurons to direct activation was comparable in SH versus GH flies (Figure S6). We next imaged MAP or fpC1 neurons while optogenetically stimulating CAP neurons. In both sexes, the response of MAP/fpC1 neurons to CAP activation was significantly greater in SH than in GH flies (Figure 7A). These results suggest that the CAP→MAP functional connection in males, and the CAP→fpC1 functional connection in females, may be strengthened by social isolation.

To examine the behavioral consequences of this enhanced functional connectivity, we optogenetically activated CAP neurons in SH flies of both sexes (Figures 7B and 7C), at the lowest photostimulation intensity used for GH flies in our titration experiment (Figure 4A). SH flies exhibited increased approach behavior in both sexes, in comparison to GH flies (Figures 7Bi and 7Ci, GH→SH, 'ChR'). More importantly, in both sexes, weak activation of CAP neurons in SH flies triggered both approach and sex-specific attack behaviors (Figures 7Bii and 7Cii, GH→SH, 'ChR'), a phenotype not observed in GH flies of either sex at this stimulation intensity (Figures 7Bii and 7Cii, GH→GH, 'ChR'). Thus, in SH males, weak activation of CAP neurons promoted both approach and lunging, a response not observed in GH males even at the highest photostimulation intensity tested (Figure 4Aiv). Taken together, our *in vivo* imaging analyses and behavioral experiments support the conclusion that social isolation elevates aggressiveness in both sexes, at least in part, by strengthening the functional connectivity between CAP neurons and their downstream MAP/fpC1 targets.

Discussion

How behaviors that exhibit sexually monomorphic, as well as dimorphic, action components are implemented in the brain is poorly understood. Here we have identified three cell types that regulate aggression in *Drosophila*: one type is sexually shared, and the other two are sex-specific. The shared cell type, called CAP neurons, mediates aggressive approach in both sexes, and in turn activates the dimorphic cell types, called MAP in males and fpC1 in females, which control dimorphic attack (Figure 7D). These mirrored circuit motifs therefore underlie the divergence of male and female aggressive behaviors, from their monomorphic appetitive/motivational to their dimorphic consummatory phases. This circuit logic may generalize to other behaviors and organisms.

Dissociable neural control of aggressive approach vs attack

The results presented here suggest that in both sexes, the approach vs. attack phases of aggression are controlled by different neuron types. Importantly, the approach behavior controlled by CAP neurons is directed preferentially towards same-sex conspecifics, and is required for natural attack, arguing that it expresses aggressive motivation, and not simply generic social investigation. However, this conclusion presents a seeming paradox: if approach behavior is required for attack during natural aggression, how can experimental MAP stimulation cause attack without promoting approach behavior as well?

Close examination of fighting patterns during MAP stimulation suggests an explanation for this paradox. Tester males lunged during MAP stimulation only when the target fly was in close proximity. Such proximity resulted from both directed approaches, and through serendipitous close encounters, such as when a climbing target fly fell off the chamber wall next to the tester (Video S1). The relatively small arenas (16mm in diameter) used in our experiments increased the frequency of the latter type of events. Indeed, the majority (75%) of lunge bouts promoted by MAP stimulation occurred following such serendipitous close encounters. Moreover, once an initial MAP-evoked lunge occurred, tester males could perform lunges continuously towards the target as long as it remained in proximity (Figure S5D). In this way, MAP stimulation can promote lunging without also promoting approach.

We find that approach and attack are triggered at progressively higher stimulation intensities. Other studies have shown that a single pair of descending neurons controls sequential male courtship actions in a ramp-to-threshold manner (McKellar et al., 2019). Our observations are consistent with such a mechanism operating to control the transition from approach to attack, but further studies will be required to validate this hypothesis (Figure 7E). Similarly, in mammals, optogenetic stimulation of estrogen receptor 1-positive ($Esr1^{+}$) neurons in the ventrolateral subdivision of the ventromedial hypothalamus (VMHvl) has been shown to promote sniffing/mounting and attack at low vs. high thresholds, respectively (Lee et al., 2014). However calcium imaging has revealed substantial overlap between attack- vs. sniff-tuned $VMHvl^{Esr1+}$ neurons (Remedios et al., 2017). In contrast, distinct populations of neurons in the lateral hypothalamus (LH) have been shown to be active during the appetitive vs. consummatory phases of feeding behavior (Jennings et al., 2015). Whether these distinct populations are functionally interconnected, and whether they control their respective behaviors at different thresholds, is not yet clear.

Physiological sexual dimorphisms in anatomically similar circuits

Despite the overall similarity in circuit logic between males and females, the light-intensity threshold for optogenetically induced CAP-mediated approach is lower in females. Our data indicate that this difference may reflect, at least in part, a higher intrinsic excitability of CAP cells in GH females than in males. This difference may also explain why wild-type GH females are more aggressive than wild-type GH males (Figures 1Bi and 1Ci, GH), although further studies are required to confirm this.

In addition, the intensity threshold for attack stimulated by optogenetic activation of CAP cells is lower in females than in males, particularly in GH flies where stimulation of CAP neurons in males does not promote attack at all (Figure 4A). In SH flies, however, attack as well as approach could be evoked by CAP stimulation in both sexes (Figures 7B and 7C). One explanation for this sex difference is that in GH males, the threshold for MAP activation by CAP cells is higher than the threshold for fpC1 activation in females. In SH males, however, because the excitability of the CAP→MAP connection is enhanced, this threshold

can be reached and therefore lunging can be evoked by CAP stimulation (Figures 7Ai and 7Bii). The synaptic basis for this physiological dimorphism remains to be investigated.

Dimorphic but homologous pC1 neuron subtypes may control dimorphic aspects of aggressive behavior

The symmetry between the CAP→MAP circuit in males and the CAP→fpC1 circuit in females raises the question of whether MAP and fpC1 neurons are sex-specific, analogous cell types. pC1 neurons, a large (~50-cell) cluster of *doublesex*-expressing cells labeled by NP2631-Gal4 (Koganezawa et al., 2016), promote both male and female aggression (Ishii et al., 2020; Koganezawa et al., 2016). More recent studies have suggested that a sex-specific subpopulation of pC1 neurons, called pC1d, controls aggression in females (Deutsch et al., 2020; Palavicino-Maggio et al., 2019; Schretter et al., 2020; Wang et al., 2020). These cells resemble fpC1 cells (Figure 6Bi), although establishing correspondence between these pC1 subtypes is currently challenging, due to Gal4 driver incompatibility and the multiplicity of morphologically similar cell types within a cluster.

Our data suggest that MAP neurons may represent a male-specific subclass of pC1 neurons. Although MAP neurons are not labeled by NP2631-Gal4 (Figure S4D), the cell body location and the morphology of these cells appear similar to that of some pC1 neurons (Figure 2Eiv), an observation supported by NBlast analysis (Figure S4C). MAP and fpC1 neurons share their main projection patterns, but the latter have two extra branches projecting laterally and ventrally (Figures 6Ai and 6Bi). This suggests that these two cell types are indeed functional homologues. This point should be further clarified once an EM-level connectome of the male fly brain is available.

Relationship to other aggression-promoting neurons

While our data provide an overall logic for the functional organization of male vs. female aggression circuits, they leave out the details of its implementation. For example, it is not yet clear whether CAP→MAP/fpC1 connections are direct or indirect, and whether they are

exclusively feed-forward or also recurrent. Our initial examination of the female EM connectome identified several candidate CAP-like neurons, some of which make direct monosynaptic connections with fpC1-like pC1d neurons. pC1d neurons in turn connect to aIP-g neurons, which also promote female aggression; however there is extensive recurrence between pC1d and aIP-g cells (Deutsch et al., 2020, Schretter et al., 2020).

Although a male EM connectome is not yet available, several other groups of male-specific neurons, such as the FruM^{+} Tachykinin (Tk)-expressing neurons, have been previously shown to play important roles in male aggression (Asahina et al., 2014; Hoopfer, 2016; Kravitz and Fernández, 2015). Whether and how these male-specific neurons interact with CAP and MAP neurons to regulate male aggression remains largely unexplored. Initial epistasis experiments suggest that Tk neurons act downstream of the Eb5 (CAP+MAP) neurons to regulate aggression (Figure S7). In the future, it will be interesting to clarify the implementation of connectivity between CAP, MAP, and Tk neurons once a male connectome becomes available. Nevertheless, the functional pathways defined by CAP and MAP/fpC1 neurons establish a conceptual framework for understanding how monomorphic and dimorphic aggressive behaviors are regulated in the two sexes.

Dimorphic aggressive behavior and internal states

A fundamental question that emerges from the sexual dimorphism in fly aggressive behavior (Nilsen et al., 2004; Vrontou et al., 2006) is whether the central motive state of aggressiveness is encoded similarly or differently in the two sexes. Our data suggest that CAP neurons might be candidates for controlling an internal state of aggressiveness that is common to both sexes. Two lines of evidence support this idea. Firstly, CAP stimulation promotes the appetitive phase of aggression (Figure 3B). In other systems, the appetitive phase of goal-directed behaviors has been shown to reflect an internal state of motivation or drive (Gentry et al., 2019; Jennings et al., 2015; Salamone and Correa, 2012). Secondly, in SH flies CAP neurons are able to evoke not only approach, but also attack, in both sexes. The ability of CAP neurons to control different phases of aggression, in a scalable manner (Figure 7E), is reminiscent of the function of VMHvl^{Esr1+} neurons in mice (Falkner et al.,

2014; Falkner et al., 2016; Lee et al., 2014; Remedios et al., 2017; Yang et al., 2013); these cells have been suggested to mediate an aggressive internal state (Anderson, 2016; Anderson and Adolphs, 2015; Hashikawa et al., 2016).

Finally, our findings raise the question of whether sexually dimorphic attack neurons, analogous to MAN and fpC1, are present in other species. In rodents, males and females attack different body parts of a same-sex intruder (Blanchard et al., 1975; Sgoifo et al., 1992). This topographic difference in biting may reflect sexual dimorphisms in other aspects of rodent aggression that have not yet been fully characterized. Whether this behavioral dimorphism reflects underlying sex differences in the neural control of attack is also unclear. In mice, Esr1/progesterone receptor-expressing ($Esr1^{+}/PR^{+}$ neurons) glutamatergic neurons in VMHvl have been shown to control both male and maternal aggression (Hashikawa et al., 2017; Lee et al., 2014; Yang et al., 2013). However, recent single-cell RNA sequencing data have revealed at least 7 distinct transcriptomic cell types among $VMHvl^{Esr1+/PR+}$ neurons, some of which are male- or female-specific (Kim et al., 2019). Several of these cells types are specifically activated during aggression, including one that is male-specific. Which transcriptomic cell type(s) is activated during maternal aggression is not yet clear. Thus, whether the same or different cell types regulate male vs. female aggression in vertebrates, as well as how different phases of aggressive behavior are regulated in each sex, remains to be determined.

Main Figures

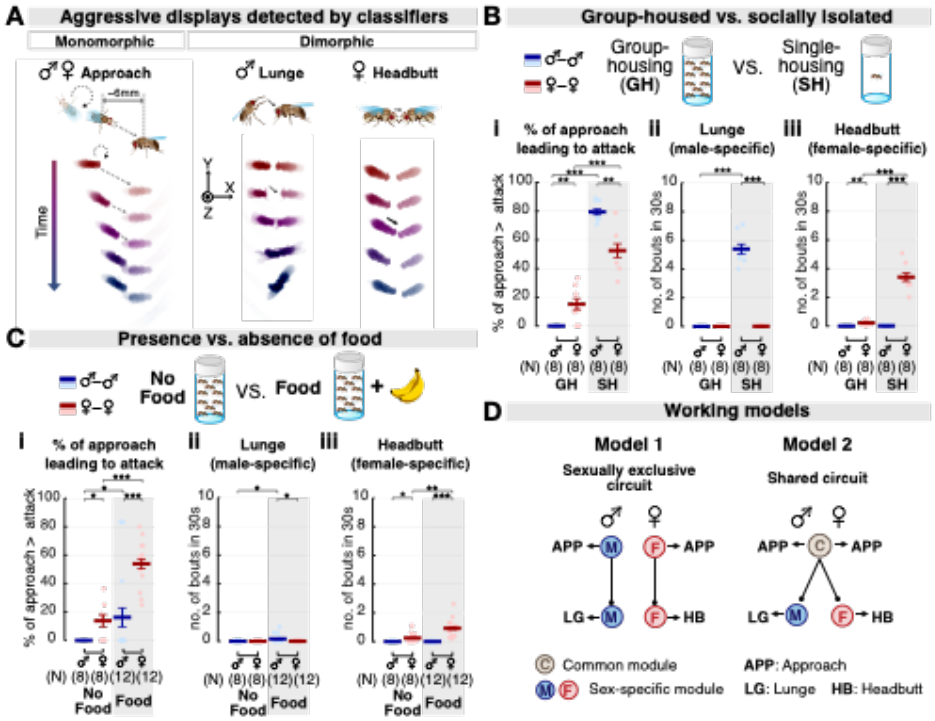


Figure 1. Contextual influences on sexually monomorphic and dimorphic aggressive actions in males and females

(A) Example bouts detected by automated behavioral classifiers. Approach: fly orients and moves towards target. Lunge: fly raises its upper body and slams down onto target. Headbutt: fly thrusts its body towards the target and strikes it with its head. See also Figure S1.

(B-C) Effects of social isolation (B) and food presence (C) on male vs. female aggression. (B) Testers were reared either in groups (GH; 20 flies/vial) or in isolation (SH; 1 fly/vial) for 6 days prior to test. In (C), a banana chunk ("Food") was provided during testing of GH flies. Dark lines: mean±SEM. Light circles: individual data. Here and throughout, ns, not significant; *p<0.05; **p<0.01; and ***p<0.001. Full genotypes of experimental flies for this and subsequent figures are listed in Table S1. Statistical data are listed in Table S3.

(D) Proposed models for aggression circuitry in males and females. Model 1, the aggression circuit in each sex is composed exclusively of sex-specific components. Model 2, some aggression circuit components, controlling similar behaviors, are shared by both sexes.

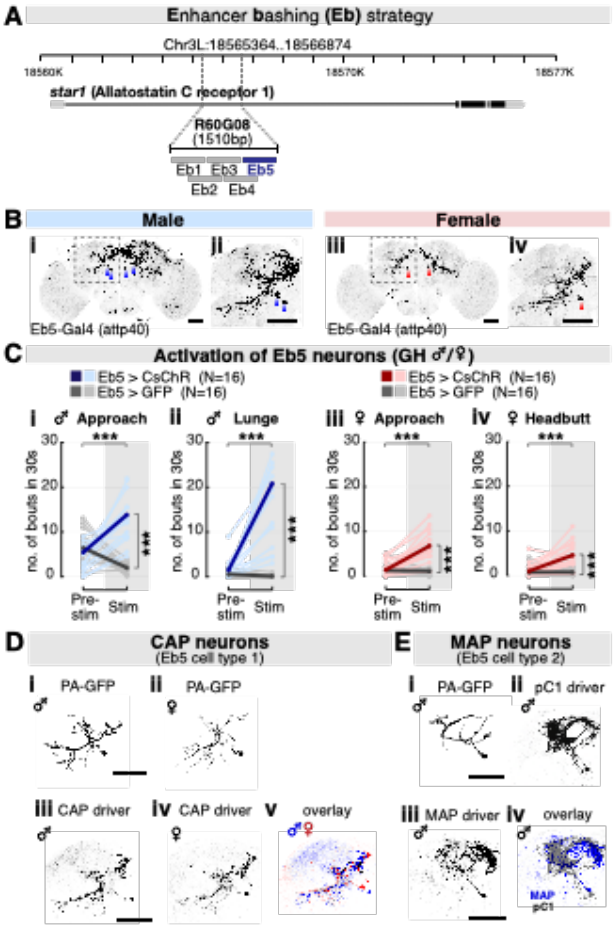


Figure 2. Identification of sexually monomorphic and dimorphic aggression-promoting cell types

(A) "Enhancer-bashing" strategy to identify GAL4 drivers for R60G08 subpopulations.

(B) Neurons labeled by Eb5-Gal4 (attp40) in males (i-ii) and females (iii-iv). Areas outlined by dashed boxes in (i) and (iii) are enlarged in (ii) and (iv), respectively. Arrowheads: cell body locations. Scale bar: 50µm. See also Figures S3A and S4B.

(C) Activation of Eb5 neurons strongly promotes male (i-ii) and female aggression (iii-iv). Shown are bouts of approach (i and iii), lunging (ii) and headbutting (iv). Light and dark color lines: individual data and the mean, respectively. See also Figure S3B.

(D) Morphology of the sexually shared CAP neurons in males and females. Neuronal traces labelled by activated photo-activatable (PA) GFP (i-ii), CsChrimson expression driven by CAP driver (iii-iv; Eb5-Gal4 (attp40); R22F05-Gal80(attp2)), and overlay between sexes (v). Scale bar: 50µm. See also Figure S3C-E.

(E) Morphology of the male-specific MAP neurons in males. Neuronal traces labelled by activated PA-GFP (i), CsChrimson expression driven by pC1 driver (ii; NP2631/dsx-Flp), or by MAP driver (iii; Eb5-AD (vk27), R22F05-DBD(attp2)) and overlay between pC1 and MAP (iv). Scale bar: 50µm. See also Figure S4A-D.

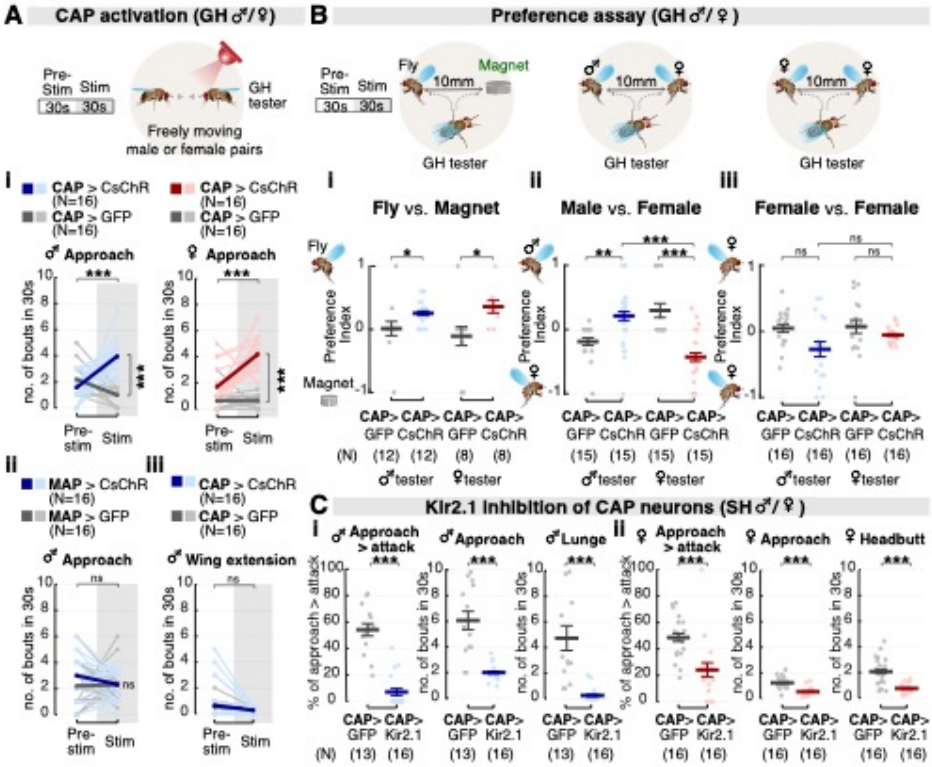


Figure 3. CAP stimulation evokes aggressive approach in males and females

(A) CAP activation in GH males and females (i), but not MAP activation in males (ii), promotes approach. Courtship, measured by bouts of wing extension, is not affected by CAP stimulation (iii). Light and dark color lines: individual data and the mean, respectively.

(B) Analysis of approach preference in CAP-stimulated testers towards: (i) flies vs. an inanimate object (magnet); (ii) same- vs. opposite-sex fly targets; and (iii) two female fly targets. The preference index (PI): (i) PI = ($n_{\text{toward the fly target}} - n_{\text{toward the magnet}}$) / n_{total}; (ii), PI = ($n_{\text{toward the male target}} - n_{\text{toward the female target}}$) / n_{total}; (iii), PI = ($n_{\text{toward the left female target}} - n_{\text{toward the right female target}}$) / n_{total}. See also Methods. Dark lines: mean±SEM. Light circles: individual data.

(C) Silencing of CAP neurons using Kir2.1 reduces naturally occurring aggression in SH males (i) and females (ii). Dark lines: mean±SEM. Light circles: individual data.

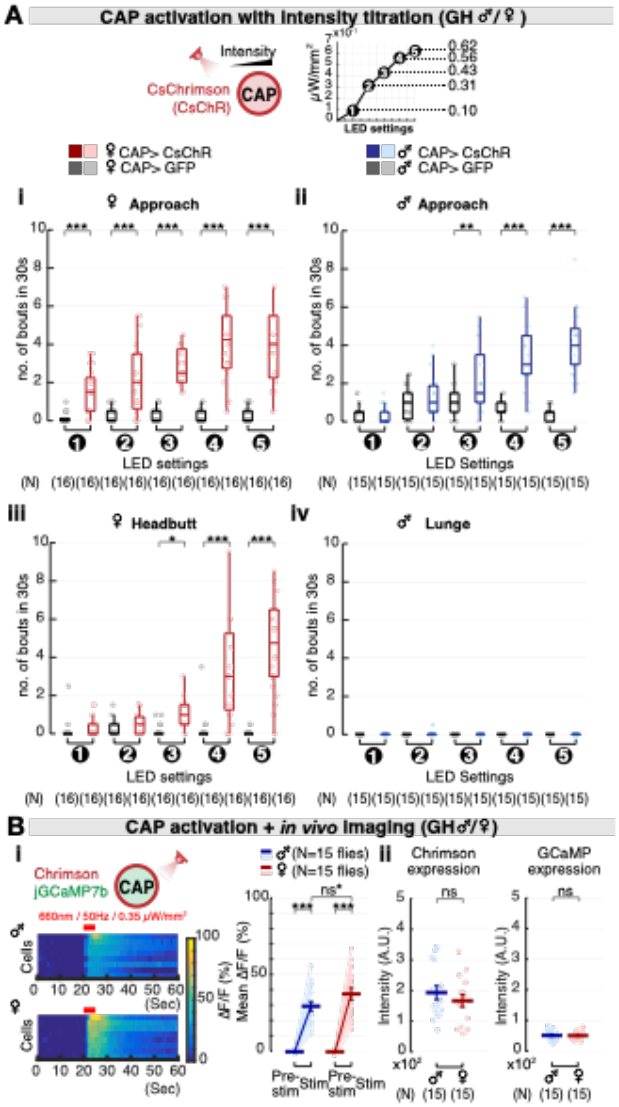


Figure 4. Aggression-promoting thresholds for CAP activation differ in males and females

(A) Optogenetic activation of CAP neurons at five increasing intensities of photostimulation in females (i, iii) and males (ii, iv), with dependent variables of approach (i, ii) headbutt (iii) or lunge (iv). The intensities of the stimulation (❶-❺) correspond to 0.1, 0.31, 0.43, 0.56, 0.62 μW/mm^2. Light circles: individual data.

(B) (i) GCaMP fluorescence changes (ΔF/F) in CAP neurons in response to photo-simulation of the same cells, in males versus females. Red bar: 5 seconds of 660nm photostimulation. Light circles: individual data. Dark lines: mean±SEM. ns*: non-significant after the Bonferroni correction. (ii) Expression level of Chrimson::tdTomato or GCaMP7b in male and female CAP neurons, measured by fluorescence intensity.

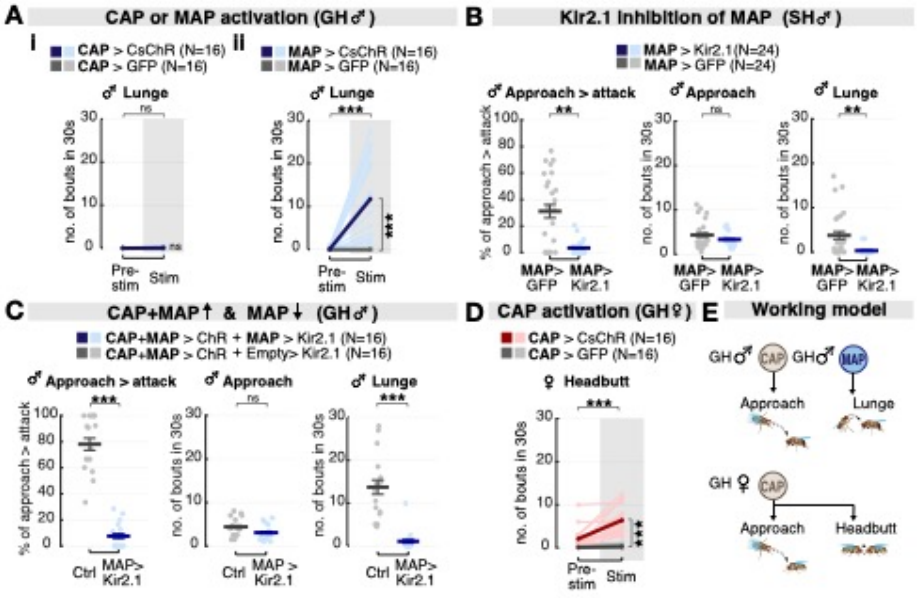


Figure 5. MAP and CAP stimulation elicits dimorphic attacks in males and females, respectively

(A) Optogenetic stimulation of MAP neurons alone, but not of CAP neurons, is sufficient to evoke lunging in GH males. Light and dark colored lines: individual data and the mean, respectively.

(B) Kir2.1 inhibition of MAP neurons in SH males reduces spontaneous lunges but does not affect approaches towards a male target. Dark lines: mean±SEM. Light circles: individual data.

(C) MAP silencing suppresses lunging promoted by co-activation of CAP and MAP cells, but does not reduce approach, in GH males. Dark lines: mean±SEM. Light circles: individual data.

(D) CAP stimulation is sufficient to evoke headbutting in GH females. Light and dark color lines: individual data and the mean, respectively.

(E) Summary of the behavioral phenotypes produced by CAP or MAP stimulation in GH flies. In males (upper), CAP stimulation promotes approach, whereas MAP stimulation triggers lunging. In females (lower), CAP stimulation promotes approach and headbutting.

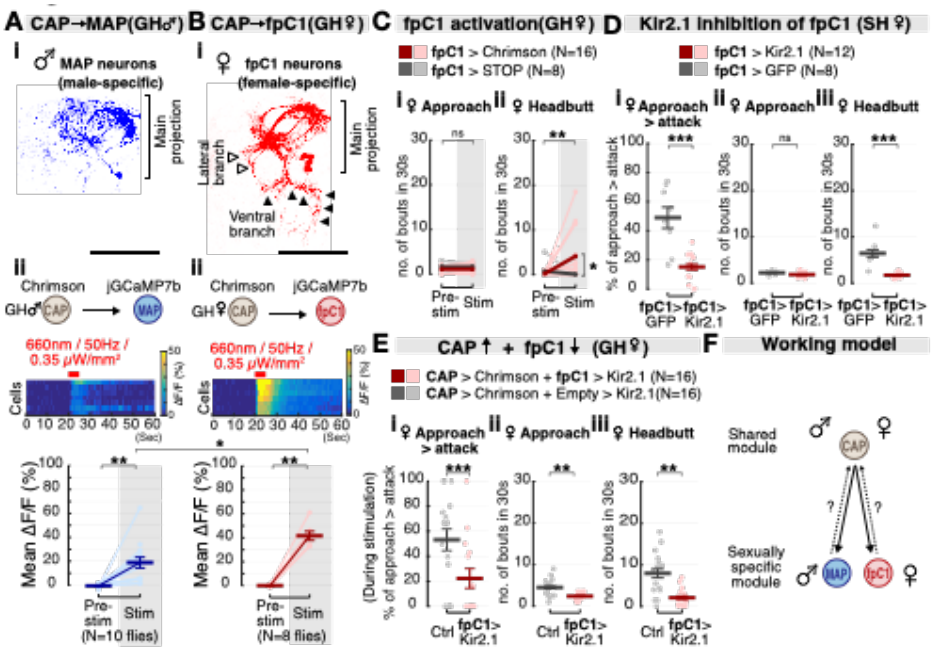


Figure 6. Functional connectivity between monomorphic and dimorphic circuit modules

(A) Morphology of MAP neurons (i) and jGCaMP7b fluorescence changes (ii) in response to CAP stimulation in GH male flies. Light circles: individual data. Dark lines: mean±SEM. Fig. 6Ai is duplicated from Fig. 2Eiii for purposes of comparison between MAP and fpC1 neurons. Scale bar: 50µm. Mann-Whitney U test, corrected for multiple comparisons.

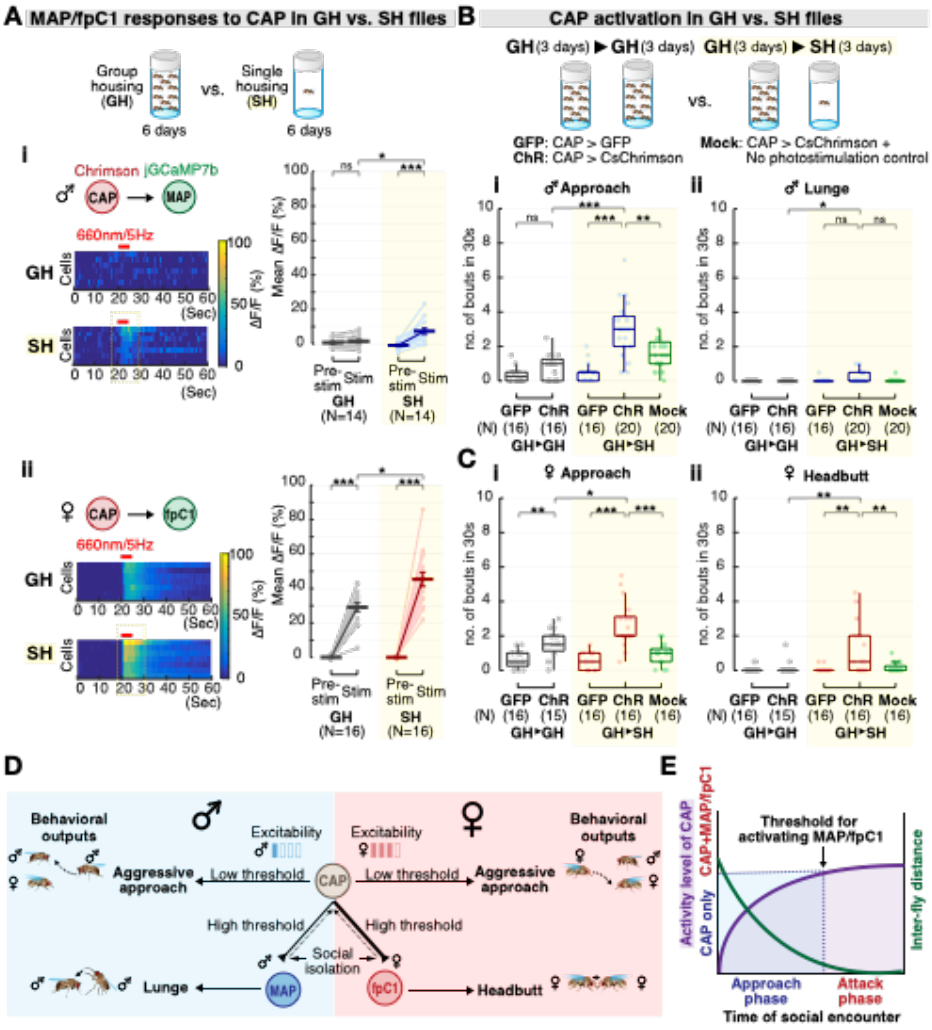
(B) Morphology of fpC1 neurons (i) and jGCaMP7b fluorescence changes (ii) in response to CAP stimulation in GH females. Light circles: individual data. Dark lines: mean±SEM. Scale bar: 50µm. Mann-Whitney U test, corrected for multiple comparisons.

(C) Optogenetic activation of fpC1 neurons promotes headbutting, but not approach, in GH females. Light and dark color lines: individual data and the means, respectively. See also Figures S4E and S5D.

(D) Inactivation of fpC1 neurons with Kir2.1 expression. Dark lines: mean±SEM. Light circles: individual data.

(E) Behavioral epistasis between the upstream CAP neurons and the downstream fpC1 neurons. Dark lines: mean±SEM. Light circles: individual data.

(F) Summary of circuit connectivity in males and females. Sexually monomorphic CAP neurons functionally connect with dimorphic MAP and fpC1 neurons in males and females, respectively. Dashed arrow: potential feedback from MAP/fpC1 onto CAP.



(Please see legend on next page)

Figure 7. Social isolation enhances aggressiveness by strengthening circuit functional connectivity

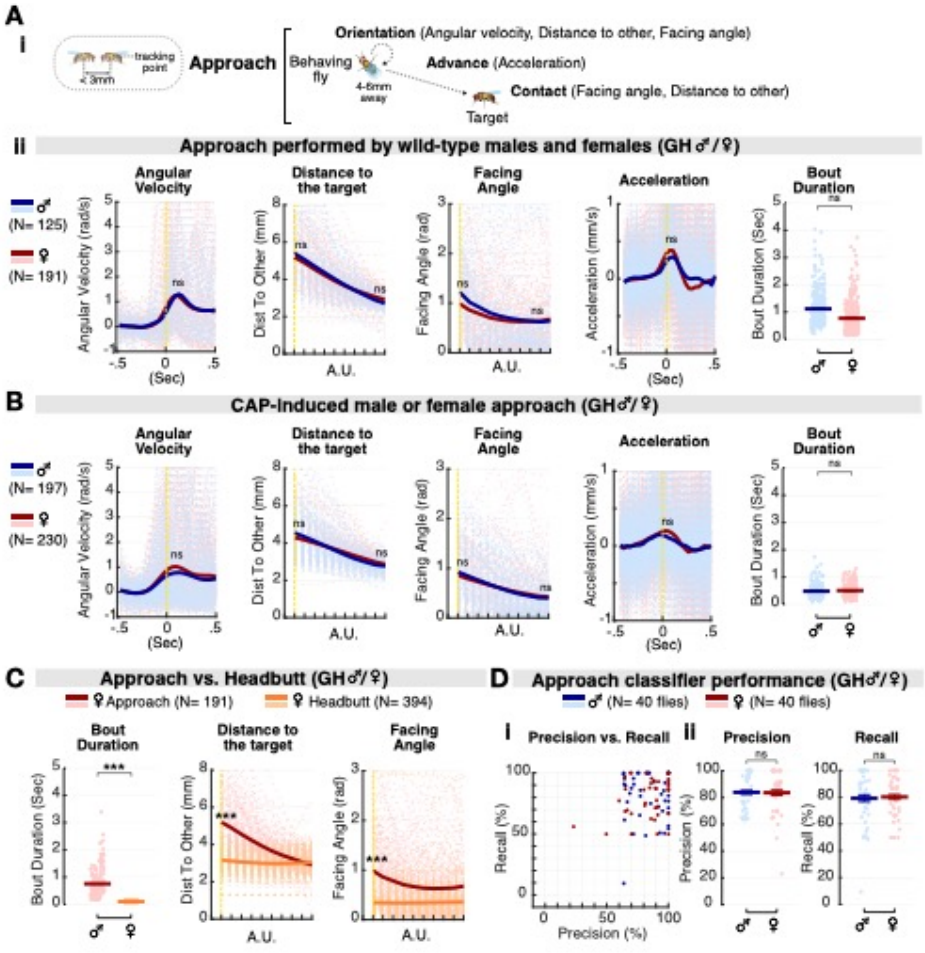
(A) jGCaMP7b fluorescence changes in MAP (i) or fpC1 neurons (ii) in response to CAP stimulation in group-housed (GH) or single-housed (SH) flies. Mann-Whitney U test, corrected for multiple comparisons. Light circles: individual data. Dark lines: mean±SEM.

(B-C) Optogenetic activation of CAP neurons in flies reared in groups for 6 days (GH>GH) or in groups for 3 days followed by isolation for 3 days (GH>SH). "GFP," CAP>GFP control; "ChR," CAP>CsChrimson with photostimulation; "Mock," CAP>CsChrimson without photostimulation. Light circles: individual data.

(D) Diagram illustrating circuit control of sexually monomorphic and dimorphic phases of aggression. Sexually shared CAP neurons control appetitive (approach) and trigger consummatory (lunge vs. headbutt) aggressive behavior via MAP or fpC1 neurons in males vs. females, respectively. Low vs. High threshold: the relative light intensities used for CAP stimulation to elicit appetitive vs. consummatory behavior, respectively. MAP or fpC1 stimulation promotes attack independently of approach. The CAP→MAP/fpC1 connectivity is a locus for experience-dependent enhancement of aggression. The synaptic connectivity underlying the functional connection between CAP and MAP/fpC1 may be direct or indirect. Dashed arrows: potential feedback from MAP/fpC1 onto CAP.

(E) Model showing how progression from the approach to attack phases of aggression might be controlled by a ramp-up in CAP activity, from below to above threshold for MAP/fpC1 activation (arrow). CAP activity is predicted to vary inversely with distance between flies, for example due to an increase in the intensity of sensory cues.

Supplemental Figures



(Please see legend on next page)

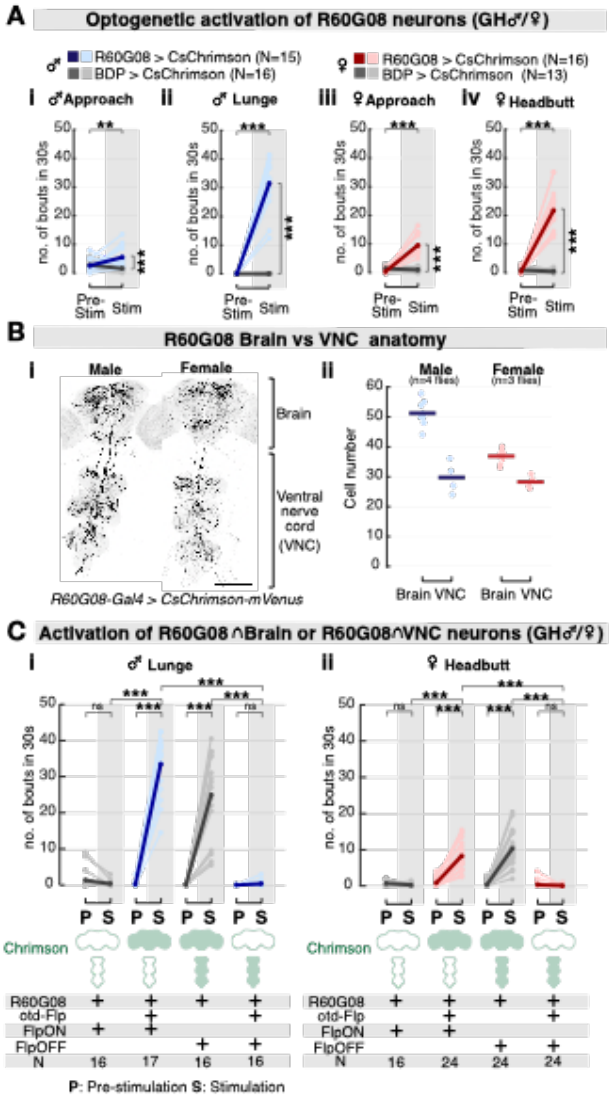
Supplemental Figure 1. Comparison between male and female approach

(A) Approach performed by wild-type males or females are comparable in the tracking features used for detecting the approach bouts and have similar durations. (i) Approach involves three motor elements: orientation, advance, and contact. (ii) Yellow dashed lines: the start of the approach bouts. For the 'Angular Velocity' and the 'Acceleration' plots, the starting frame of all approach bouts was aligned at time zero. The data between 0.5 second before and after time zero were plotted to show the increase in angular velocity and the acceleration during approach bouts. For the 'Distance to the Target' and the 'Facing Angle' plots, we rescaled the timeline of each approach bout so that the start and the end of every bout are aligned. A.U.: arbitrary unit. Dark blue and dark red lines: male mean and female mean, respectively. Light blue and light red dashed lines: individual data of males and females, respectively. ns, not significant.

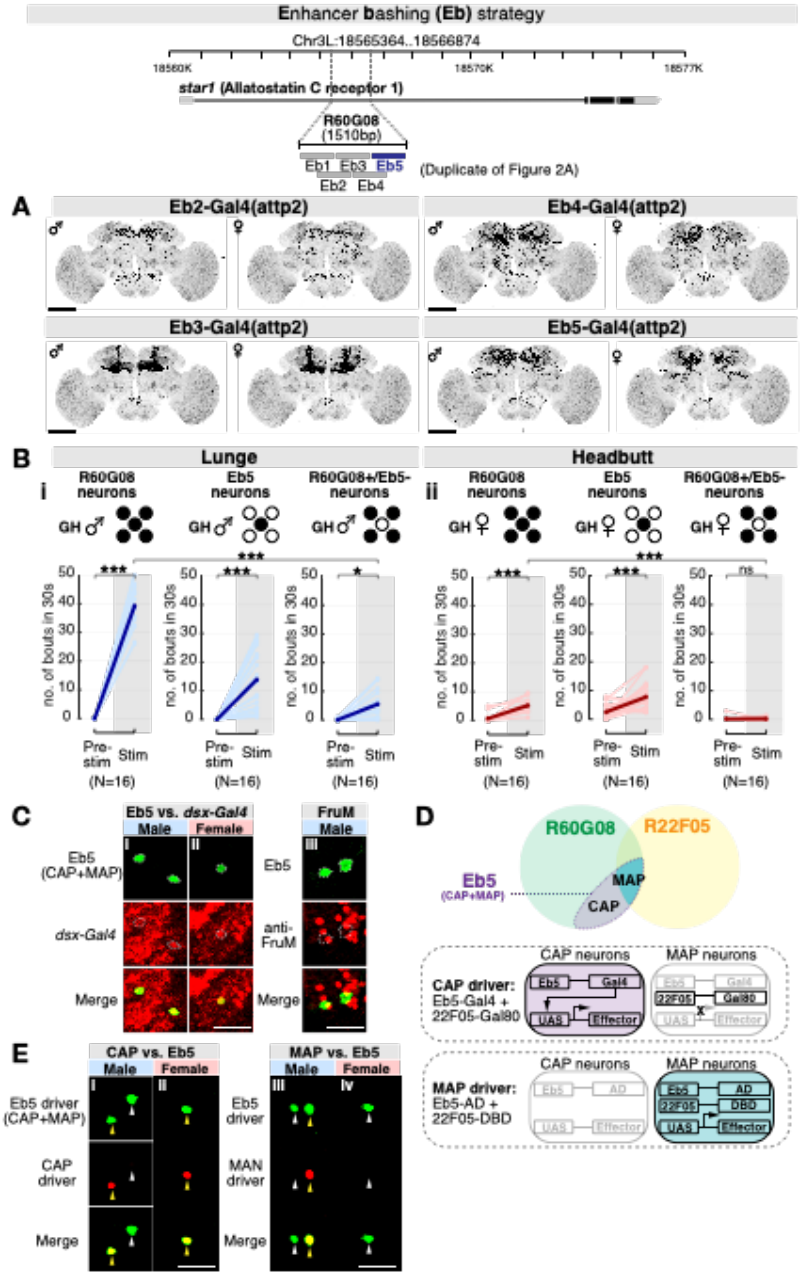
(B) Male and female approach induced by CAP activation have similar durations and are comparable in the tracking features.

(C) Approach and headbutt bouts differ significantly in 'Bout Duration', as well as 'Distance to the Target' and 'Facing Angle' at the beginning of the bouts. ***$P<0.001$.

(D) Comparison between the approach classifier performance in the recordings of the wild-type males and females. Recall (%): percentage of the true behavioral frames that were detected (True positive / (True positive + False negative)). Precision (%): percentage of the detected behavioral frames that were true positive (True positive / (True positive + False positive)). (i) The recall rate obtained from each fly is plotted against the precision rate. Blue: male data. Red: female data. Dark color lines: mean±SEM.



Supplemental Figure 2. R60G08-Gal4 neurons promote male and female aggression
(A) Optogenetic activation of R60G08-Gal4 neurons in males (i-ii) and females (iii-iv). Light and dark color lines: individual data and the mean, respectively. Here and throughout, *p<0.05; **p<0.01; ***p<0.001; and ns, not significant.
(B) Expression patterns (i) and cell counts (ii) of R60G08-Gal4 in the male and female nervous systems. Light circles: individual data. Dark bar: mean. Scale bar: 100µm.
(C) Activation of R60G08 neurons in the brain is sufficient to promote male and female aggression. Light and dark color lines: individual data and the mean, respectively.



(Please see legend on next page)

Supplemental Figure 3. Eb5 neurons mediate R60G08-induced male and female aggression

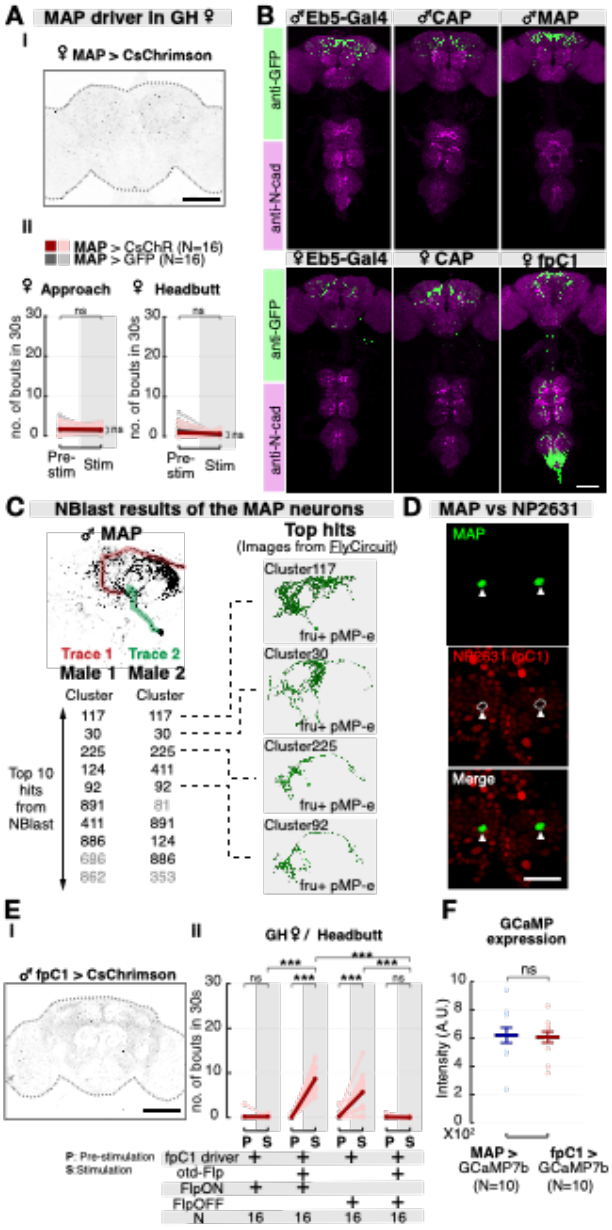
(A) Expression patterns of enhancer-bashing (Eb) Gal4 lines in males and females. Scale bar: 100μm. Eb1-Gal4 transgenic flies were excluded from analysis due to viability issue.

(B) Optogenetic activation of R60G08, Eb5, and R60G08+/Eb5- neurons in males (i) and females (ii). Light and dark color lines: individual data and the mean, respectively.

(C) CAP and MAP neurons are *doublesex* positive but *fruitless* negative. (i-ii) Chrimson::tdTomato expression driven by *dsx-Gal4* overlaps with CAP/MAP neurons in males and with CAP neurons in females. (iii). FruM signals are absent from CAP/MAP neurons in males. Scale bar: 20μm.

(D) Intersection strategy for generating the CAP and MAP drivers.

(E) Neurons labeled by the CAP and MAP drivers are subsets of Eb5 neurons. Yellow and white arrowheads: overlapping and non-overlapping cells, respectively. Scale bar: 20μm.



(Please see legend on next page)

Supplemental Figure 4. Further analysis on MAP and fpC1 neurons

(A) No detectable fluorescence signals from the MAP driver in females (i). Scale bar: 100μm. (ii) Optogenetic stimulation does not promote approach or headbutt in MAP>CsChrimson females. Light and dark color lines: individual data and the mean, respectively. ns, not significant.

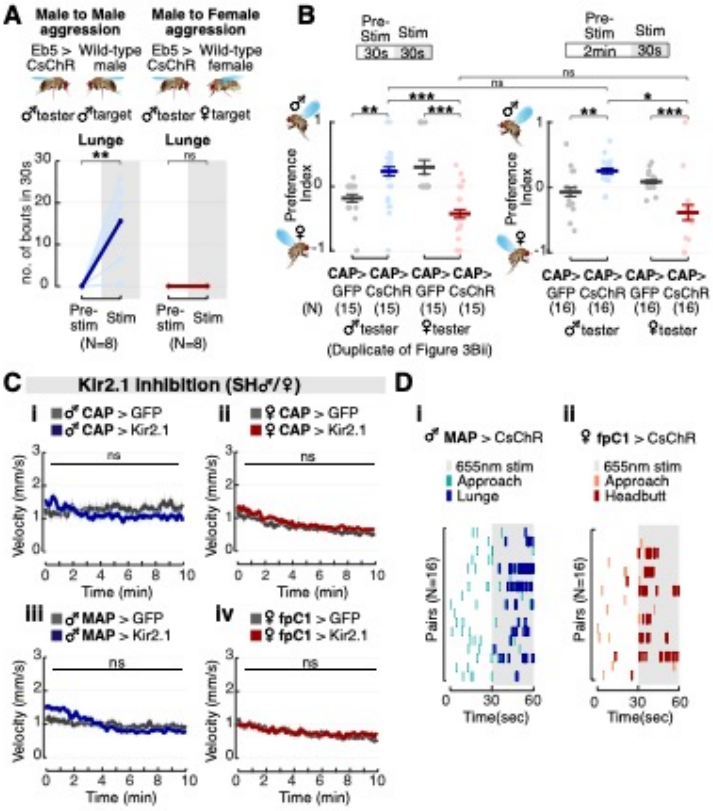
(B) The ventral nerve cord (VNC) expression of the Eb5, CAP, MAP, and fpC1 drivers. Only the fpC1 driver labels a group of neurons in the abdominal neuropil. Scale bar: 100μm.

(C) NBlast results of MAP neurons. pMP-e is a synonym for pC1 neurons (Cachero et al., 2010; Kimura et al., 2008; Yu et al., 2010). The image of MAP neurons is a duplicate of Fig 2Eiii and is provided here to facilitate comparison. Trace 1 and 2 from independent fly samples were used for blasting. FlyCircuit images were obtained from the NCHC (National Center for High-performance Computing) and NTHU (National Tsing Hua University), Hsinchu, Taiwan.

(D) Fluorescence images of the pC1 driver, NP2631, and the MAP driver show that the MAP neurons are not labeled by the pC1 driver. Scale bar: 20μm.

(E) (i) No detectable fluorescence signals from the fpC1 driver in males. Scale bar: 100μm. (ii) Activation of fpC1 neurons in the female brain are sufficient to promote female aggression. ***$p<0.001$.

(F) Comparison between the jGCaMP7b expression level driven by the MAP and the fpC1 driver. Light color dots: individual data. Dark color lines: mean±SEM.



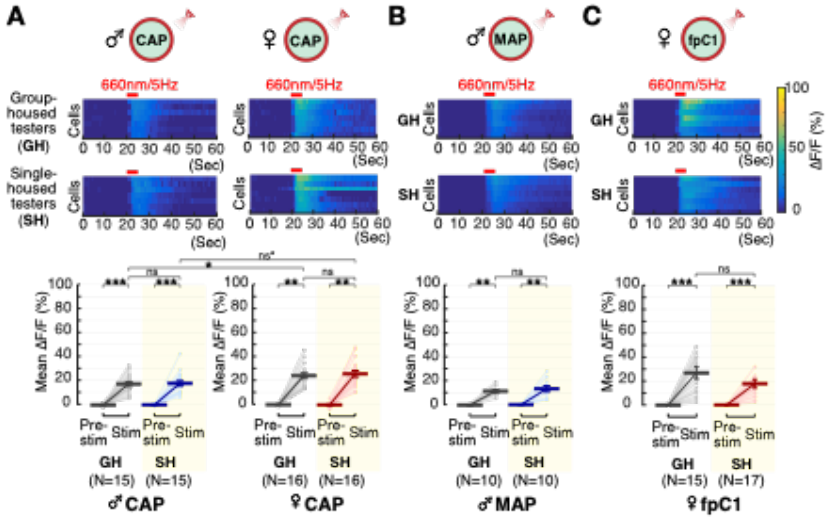
Supplemental Figure 5. CAP stimulation induces same-sex target preference independently of how long the testers interacted with the targets before stimulation and Kir2.1 expression in CAP, MAP or fpC1 neurons does not affect locomotor activity

(A) Optogenetic stimulation of Eb5 neurons in males does not promote aggression toward a freely moving female target. Light and dark colored lines: individual data and the mean, respectively. $^{**}p<0.01$; ns, not significant.

(B) An extended pre-stimulation period (2 minutes) does not change the same-sex target preference induced by CAP stimulation, in comparison to the standard pre-stimulation period (30 sec). Light circles: individual data. Dark line: mean±SEM. ns, not significant; $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

(C) Locomotor activity of the Kir2.1-expressing testers and the GFP-expressing control flies was measured as velocity changes over the 10-min behavioral test. The testers that express Kir2.1 in CAP (i-ii), MAP(iii), or fpC1 (iv) neurons showed comparable locomotor activity as the control flies. Dark color lines and light color envelopes: mean±SEM. ns, not significant.

(D) Optogenetic activation of MAP neurons in males (i) or fpC1 neurons in females (ii) promotes clusters of lunges or headbutts, respectively.



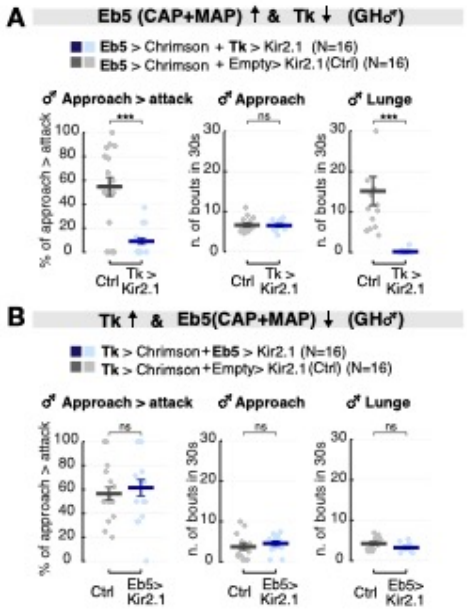
Supplemental Figure 6. Excitability of CAP, MAP, and fpC1 neurons is not affected by social isolation

(A) jGCaMP7b fluorescence changes of CAP neurons in response to photostimulation in group-housed (GH) versus single-housed (SH) males and females. Bar graphs on *left* and *right* show data from males and females, respectively. Red bar: 5 seconds of 660nm 5Hz photostimulation. Light circles: individual data. Dark bars: mean±SEM. Here and throughout, WSR test and MWU test, corrected for multiple comparisons. ns*: not significant after Bonferroni correction.

(B) jGCaMP7b fluorescence changes of MAP neurons to photostimulation in GH versus SH males.

(C) jGCaMP7b fluorescence changes of fpC1 neurons to photostimulation in GH versus SH females.

No significant difference in excitability is observed between GH and SH conditions for all above settings.



Supplemental Figure 7. Tachykinin (Tk) neurons act downstream of Eb5 neurons
(A) Male aggression induced by activation of Eb5(CAP+MAP) neurons is suppressed by Kir2.1 inhibition of Tk neurons. Aggression phenotype is measured by the percentage of approaches leading to attack as well as the numbers of approach or lunge bouts during optogenetic stimulation. Light color dots: individual data. Dark color lines: mean±SEM. ***p<0.001; and ns, not significant.
(B) Male aggression induced by Tk activation is not affected by Kir2.1 inhibition of Eb5 neurons. Light color dots: individual data. Dark color lines: mean±SEM. ns, not significant.

Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-GFP(rabbit)	Thermo Fisher Scientific	Cat#A11122; RRID: AB_221569
anti-RFP(rat)	ChromoTek	Cat#5f8-100; RRID:AB_2336064
nc82(mouse)	Developmental Studies Hybridoma Bank	Cat#nc82; RRID:AB_2314866
anti-Cadherin(extracellular domain; rat)	Developmental Studies Hybridoma Bank	Cat#DN-Ex #8; RRID:AB_528121
anti-FruM(rabbit)	Barry J. Dickson Lab; Stockinger et al., 2005	N/A
Goat anti-rabbit IgG(H+L) Alexa Fluor 488	Thermo Fisher Scientific	Cat#A11008; RRID: AB_143165
Goat anti-rat IgG(H+L) Alexa Fluor 568	Thermo Fisher Scientific	Cat#A11077; RRID: AB_2534121
Goat anti-mouse IgG(H+L) Alexa Fluor 633	Thermo Fisher Scientific	Cat#A21050; RRID: AB_141431
Goat anti-rat IgG(H+L) Alexa Fluor 647	Thermo Fisher Scientific	Cat#A21247; RRID: AB_141778
Normal Goat Serum	Jackson ImmunoResearch INC.	Cat#005-000-121; RRID: AB_2336990
Chemicals, Peptides, and Recombinant Proteins		
VECTASHIELD Antifade Mounting Media	VECTOR Laboratories	Cat#H-1000; RRID: AB_2336789
Paraformaldehyde, 16% solution, EM grade	Electron Microscopy Sciences	Cat#15710; CAS no. 30525-89-4
All trans-Retinal (powder, ≥98%)	Sigma-Aldrich	Cat#R2500; CAS no.116-31-4
Insect-A-Slip	BioQuip Products	Cat#2871B
Hydrocarbon Soluble Siliconizing Fluid	ThermoFisher Scientific	Cat#TS-42800; CAS no.474-02-4
Experimental Models: Organisms/Strains		
Drosophila: Wild-type Canton S	Heisenberg lab ; Hoyer et al., 2008	N/A
Drosophila: *R60G08-Gal4 (attp2)*	Rubin Lab	RRID:BDSC_39260
Drosophila: *pBDPGAL4U (attp2)*	Rubin Lab	RRID:BDSC_68384
Drosophila: *Eb2-Gal4 (attp2)*	This study	N/A
Drosophila: *Eb3-Gal4 (attp2)*	This study	N/A
Drosophila: *Eb4-Gal4 (attp2)*	This study	N/A
Drosophila: *Eb5-Gal4 (attp2)*	This study	N/A
Drosophila: *Eb5-Gal4 (attp40)*	This study	N/A
Drosophila: BDP-p65AD(attp40)	Rubin Lab	N/A
Drosophila: BDP-Gal4DBD(attp2)	Rubin Lab	N/A
Drosophila: *Eb5-Gal80 (attp2)*	This study	N/A
Drosophila: *Eb5-p65AD (vk27)*	This study	N/A
Drosophila: Eb5-iLexA(attp18)	This study	N/A

(Please see the remaining table content on next page)

Drosophila: R22F05-Gal4DBD(attp2)	Rubin Lab	RRID:BDSC_69772
Drosophila: *R22F05-LexADBD (attp2)*	This study	N/A
Drosophila: *R22F05-Gal80 (attp2)*	This study	N/A
Drosophila: *R26E01-Gal4 (attp2)*	Bloominton Drosophila Stock Center	RRID:BDSC_60510
Drosophila: *R26E01-p65AD (attp40)*	Bloominton Drosophila Stock Center	RRID:BDSC_75740
Drosophila: dsx-Gal4	Goodwin Lab; Rideout et al., 2010	N/A
Drosophila: *dsx*-DBD	Goodwin Lab; Pavlou et al., 2016	N/A
Drosophila: dsx-Flp	Goodwin Lab; Rezával et al., 2014	N/A
Drosophila: *NP2631; dsx-Flp*	Koganezawa et al., 2016	N/A
Drosophila: otd-nls::FLPo(attp40)	Anderson Lab; Watanabe et al., 2017	N/A
Drosophila: Tk-Gal4[1]	Anderson Lab; Asahina et al., 2014	RRID:BDSC_51975
Drosophila: *20xUAS-IVS-CsChrimson.mVenus (attp2)*	Jayaraman Lab; Klapoetke et al., 2014	RRID:BDSC_55136
Drosophila: pJFRC82-*20xUAS-IVS-Syn21-GFP-p10 (attp2)*	Rubin Lab	N/A
Drosophila: pJFRC49-*10xUAS-eGFP::Kir2.1 (attp2)*	Rubin Lab	N/A
Drosophila: pJFRC81-*10xUAS-IVS-Syn21-GFP-p10 (attp2)*	Rubin Lab	N/A
Drosophila: 20xUAS-FRT-myrTopHat2-FRT-Chrimson-tdTomato-3.1(vk5)	Rubin Lab; Duistermars et al., 2018	N/A
Drosophila: 20xUAS-FRT-Chrimson-tdTomato-3.1-FRT-myrTopHat2 (vk5)	This study	N/A
Drosophila: *20xUAS-IVS-Syn21-Chrimson-tdTomato-3.1 (su(Hw)attp5)*	Gerald M. Rubin Lab; Watanabe et al., 2017	N/A
Drosophila: *20xUAS-IVS-Syn21-Chrimson-tdTomato-3.1 (vk5)*	Gerald M. Rubin Lab	N/A
Drosophila: *UAS-C3PA-GFP (III)*	Datta et al., 2008; Ruta et al., 2010	N/A
Drosophila: *20xUAS-IVS-jGCaMP7b(vk5)*	Bloominton Drosophila Stock Center; Dana et al., 2019	RRID:BDSC_79029
Drosophila: *13xLexAop-IVS-jGCaMP7b(vk5)*	Bloominton Drosophila Stock Center; Dana et al., 2019	RRID:BDSC_80915
Drosophila: 13xLexAop2-IVS-Syn21-CsChrimson-tdTomato-3.1(vk5)	Rubin Lab	N/A
Drosophila: 13xLexAop2-IVS-Syn21-Chrimson-tdTomato-3.1(su(Hw)attp5)	Rubin Lab	N/A
Drosophila: 13xLexAop2-eGFP::Kir2.1(attp40)	Rubin Lab	N/A
Drosophila: 10xUAS-nls::tdTomato(vk22)	Rubin Lab; Jung et al., 2020	N/A
Drosophila: 13xLexAop2-nls::GFP(vk40)	Rubin Lab; Jung et al., 2020	N/A

(Please see the remaining table content on next page)

Oligonucleotides		
Eb1_F: caccatccttcccacttgagctccacagc	This study	N/A
Eb1_R: agttccattcactgtgggcaatgaaacgtc	This study	N/A
Eb2_F: caccggtgcaagatagtcaatcgttgcac	This study	N/A
Eb2_R: ctgagccaaaacacatgtgggggttattg	This study	N/A
Eb3_F: caccgacgtttcattgcccacagtgaatggaact	This study	N/A
Eb3_R: aaattgctcacagcttgacacgtccaac	This study	N/A
Eb4_F: cacccaataacccccacatgtgttttggctcag	This study	N/A
Eb4_R: ttactgactttctgagaaatcccctcg	This study	N/A
Eb5_F: caccgttggacgtgtcaagctgtgagcaattt	This study	N/A
Eb5_R: caattggtgataagtattcaaatggaattaagttaagtctacaag	This study	N/A
Recombinant DNA		
pBPGUw	Pfeiffer et al., 2008	RRID:Addgene_17575
pBPp65ADZpUw	Pfeiffer et al., 2010	RRID:Addgene_26234
pBPZpGal4DBDUw	Pfeiffer et al., 2010	RRID:Addgene_26233
pBPGAL80Uw-6	Pfeiffer et al., 2010	RRID:Addgene_26236
pattB-nsyb-MKII::nlsLexADBDo	Gao et al., 2015	RRID:Addgene_64725
pBPnlsLexA::p65Uw	Pfeiffer et al., 2010	RRID:Addgene_26230
pBPnlsLexA::p65::GADUw	This study	N/A
Software and Algorithms		
JFRCtemplate2010	Jenett et al., 2012	https://github.com/VirtualFlyBrain/DrosAdultBRAINdomains
Caltech FlyTracker	Eyjolfsdottir et al., 2014	http://www.vision.caltech.edu/Tools/FlyTracker/
Janelia Automatic Animal Behavior Annotator (JAABA)	Kabra et al., 2013	http://jaaba.sourceforge.net/
MATLAB R2014a and R2016a	MathWorks	RRID: SCR_001622
Fiji	https://fiji.sc/	RRID: SCR_002285
ApE-A plasmid Editor	M. Wayne Davis	https://jorgensen.biology.utah.edu/wayned/ape/
Computational Morphometry Toolkit (CMTK)	Masse et al., 2012	https://www.nitrc.org/projects/cmtk
Simple Neurite Tracer	Longair, M.H. et al., 2011	https://imagej.net/Simple_Neurite_Tracer
NBLAST	Costa et al., 2016	http://nblast.virtualflybrain.org:8080/NBLAST_on-the-fly/
FlyCircuit 1.2	Chiang et al., 2011	http://www.flycircuit.tw/
Adobe Photoshop CC 2015	Adobe Inc.	RRID:SCR_014199

Materials and Methods

Fly strains

Canton-S (from the lab of Dr. Martin Heisenberg) was used as the wild type. Please see Table S1 for the full genotypes of flies used in each figure and see the Key Resource Table for the source of these flies. Briefly, R60G08-Gal4(attp2), pBDPGal4U(attp2), BDP-p65AD(attp40), BDP-Gal4DBD(attp2), R22F05-Gal4DBD(attp2), 20xUAS-IVS-Syn21-GFP(attp2), 10xUAS-eGFP::Kir2.1(attp2), 10xUAS-IVS-Syn21-GFP(attp2), 20xUAS-FRT-myrTopHat2-FRT-Chrimson::tdT3.1(vk5), 20xUAS-IVS-Syn21-Chrimson::tdT3.1 (su(Hw)attp5 and vk5), 13xLexAop2-CsChrimson::tdT3.1(vk5), 13xLexAop2-Chrimson::tdT3.1(su(Hw)attp5), 13xLexAop2-eGFP::Kir2.1(attp40), 10xUAS-nls::tdTomato(vk22), 10xUAS-nls::GFP(vk40) were kindly shared by the Gerald Rubin laboratory (HHMI Janelia Research Campus) and Barret Pfeiffer. *dsx*-Gal4, *dsx*-Flp, *dsx*-DBD were kindly shared by the Stephen Goodwin laboratory (University of Oxford, UK). NP2631; dsx-Flp was kindly shared by the Daisuke Yamamoto laboratory (Tohoku University, JP). R26E01-Gal4(attp2)(RRID:BDSC_60510), R26E01-p65AD(attp40)(RRID:BDSC_75740), 20xUAS-IVS-CsChrimson-mVenus(attp2) (RRID:BDSC_55136), 20xUAS-IVS-jGCaMP7b(vk5) (RRID:BDSC_80907), 13xLexAop-IVS-jGCaMP7b(vk5) (RRID: BDSC_80915) were obtained from the Bloomington Stock Center (Indiana University).

Rearing conditions

Stocks and crosses were reared at 25ºC and 50% humidity and maintained on a 12hr:12hr light:dark cycle. To keep the fly density consistent across experiments, each cross was set up with 10-12 virgin females and 5-6 males, and was flipped every two days. Experimental flies were collected mostly as virgins on the same day of eclosion and reared in isolation (single-housed (SH) condition; one fly per vial), or in groups (group-housed (GH) condition; ~20 single-sex flies per vial). For optogenetic experiments, flies were transferred to vials containing retinal food (regular fly food mixed with all-trans-retinal to a final concentration

of 0.2 mM) after collection and reared in the dark for 5-6 days. Flies were flipped to fresh retinal food vials one day before a behavioral test.

Construction of transgenic animals

Several strains were generated for this study: Eb2-Eb5-Gal4s, Eb5-p65AD, Eb5-Gal80, R22F05-Gal80, R22F05-LexADBD, and Eb5-iLexA (improved LexA). Enhancer-bashing fragments, Eb2-Eb5, were PCR-amplified from R60G08 sequence (Jenett et al., 2012; Pfeiffer et al., 2008) and cloned into pBPGUw (Addgene #17575) via Gateway and the LR reaction. A detailed description of the cloning strategy can be found in Pfeiffer et al., 2008. Sequences of the primers used for PCR amplification are listed in the key resources table. The Eb5 fragment was subcloned into pBPp65ADZpUw (Addgene #26234) and pBPGAL80Uw-6 (Addgene# 26236) to generate Eb5-p65AD and Eb5-Gal80, respectively. To make the construct R22F05-Gal80, the R22F05 fragment was first amplified from the genomic DNA of the reference strain (Adams et al., 2000) using the primers listed in the 'Janelia_info' excel sheet (Jenett et al., 2012; downloaded from Bloomington Stock Center webpage: https://bdsc.indiana.edu/stocks/gal4/gal4_janelia.html) and then cloned into pBPGAL80Uw-6 via Gateway and the LR reaction. To improve the strength of LexA, we fused the activating domain of Gal4, GAD, to the C-terminus of nlsLexA::p65 (Addgene #26230) to make pBPnlsLexA::p65::GADUw. The GAD sequence was amplified from the pBPGUw. It has been shown that the expression of a targeted gene can be enhanced by adding additional activating domains to its transcription activator (Chavez et al., 2016). The Eb5 fragment was then cloned into pBPnlsLexA::p65::GADUw via Gateway and LR reaction to create Eb5-iLexA. All constructs were verified by sequencing. To make the construct R22F05-LexADBD, we first generated the pBPZpnlsLexADBD construct. A nlsLexADBD fragment was PCR-amplified from pattB-nsyb-MKII::nlsLexADBDo (Addgene #64725) and cloned into pBPZpGal4DBD (Addgene #26233) plasmid using KpnI and HindIII sites. The R22F05 enhancer fragment was then cloned into pBPZpnlsLexADBD via Gateway and LR reaction.

Immunohistochemistry and brain registration

Brains or ventral nerve cords of 6-8 day old flies were dissected in cold PBS and fixed in 4% paraformaldehyde for 1 hour at 4ºC. After fixation, samples were washed twice with 0.05% PBST (PBS containing 0.05% Triton X-100) for 15 minutes at room temperature (RT), and incubated in 2% PBST (PBS containing 2% Triton X-100) at RT for 30 minutes. Samples were then blocked in 5% normal goat serum (NGS) solution for 2 hours or overnight at 4ºC. After blocking, samples were incubated with the primary antibody solution for two days at 4ºC. The dilution ratios for the primary antibodies used in this study were 1:1000 for anti-GFP or anti-RFP and 1:20 for nc82 or anti-NCad. To obtain even labeling of the neuropils, which is critical for subsequent brain registration, samples were transferred to freshly diluted primary antibody solution on the second day of incubation. Afterwards, the samples were washed with 0.05% PBST for 30 minutes at 4ºC three times and then incubated with the secondary antibody solution for two days at 4ºC. All secondary anti-bodies used in this study were diluted 1:1000. Finally, the samples were washed three times with 0.05% PBST for 30 minutes at 4ºC and incubated overnight in the mounting media Vectashield (Vectorlabs, Inc.) at 4ºC.

Image stacks were obtained using confocal microscopy (Olympus Fluoview FV1000 or FV3000). Representative images illustrating the expression patterns of each driver were chosen from among 6-10 dissected samples. Brain images were first registered to JFRCtemplate2010 (Jenett et al., 2012) using the CMTK registration GUI (Jefferies et al., 2007; Masse et al., 2012) and z-projected with maximum intensity under Fiji. To create overlays of two registered brain images, images were pseudocolored and layered in Photoshop.

Behavioral assays

Details for each of the five different assays performed in this paper are listed below. In general, all experiments were performed in a room maintained at 25ºC and 50% humidity. The behavioral chamber is a 12mm-high 16mm-diameter acrylic cylinder with a clear top and floor. The wall and the lid of the chambers were coated with Insect-A-Slip and silicon fluid, respectively. The floor was covered with freshly prepared apple juice agar (2.5% (w/v)

sucrose and 2.25% (w/v) agarose in apple juice) and illuminated with an 850nm backlight (SOBL-200x150-850, SmartVision Lights, Muskegon, MI). Flies were tested on the 6th or 7th day after eclosion. Flies were introduced into the chambers by gentle mouth pipetting and allowed to settle for at least two minutes before the tests began. Behaviors were recorded at 30fps from the top using a Point Grey Flea3 camera with a long pass IR filter (780 nm, Midwest Optical Systems).

Group-housed (GH) vs. single-housed (SH) assay. Canton-S males and females were collected on the day of eclosion and reared in the single-housed (one per vial; SH) or group-housed (twenty single-sex flies per vial; GH) condition. In each behavior chamber, we paired same-sex flies from different vials to avoid the influence of prior life history, e.g. a GH male from the first vial 1 was paired with a GH male from the second vial. Interactions were recorded for 10 minutes.

Food vs. no food assay. Canton-S males and females were collected on the day of eclosion and reared in group-housed condition until the test day (the 6th day after eclosion). Same-sex flies were paired in the chambers with or without a freshly prepared banana chunk (~2mm^3) in the center as the food resource, where indicated.

Optogenetic stimulation assay. Experimental flies were group-housed and raised on retinal food in the dark until the test day. Flies were transferred to fresh vials one day before the experiment. A detailed description of the photostimulation setup can be found in Inagaki et al., 2014. Briefly, a high-powered 655nm LED was mounted ~8cm above the chamber at a 24º angle to provide photostimulation at various intensities and frequencies. With the exception of the stimulation titration experiments shown in Figure 4, 10 Hz and 5Hz pulsed light with a maximum intensity = 0.62 μW/mm^2 were used to activate neurons in males and females, respectively. The stimulation protocol included a 30s baseline activity recording, two 30s stimulation blocks separated by a 30s interstimulation interval, and a 30s post-stimulation period. Behaviors observed during experiments were present as the average from two stimulation blocks. For the stimulation titration experiment shown in Figure 4A, the same set of experimental flies were tested with 5 different intensities of photostimulation

(0.1-0.62 μW/mm^2) using the same stimulation paradigm. The order of the intensity was randomly applied and each experiment was followed by a 5min interval to allow the experimental flies to recover to their baseline activity. Data were collected from two independent sets of experimental flies.

Preference assay (Figure 3B). The chamber used was a 12mm-high cylinder with a 16mm diameter. The two targets were mounted at the opposite sides of the floor, ~10mm apart using a UV glue tool kit (Bondic). For Figure 3Bi, the left and the right targets were a fly (same-sex as the tester) and a fly-size magnet (K&J Magnetics, Pipersville, PA; Cylinder 1/16"x1/16", D11-N52), respectively. The fly targets were 5-6-day old Canton-S virgin males or females frozen at -80ºC for 20 minutes immediately before the experiment. For Figure 3Bii, the male and the female fly targets were mounted at the left and the right sides of the chamber, respectively. For Figure 3Biii, the left and right targets were both females. Testers were introduced into the chamber from the top and were allowed to freely explore the arena and the targets for two minutes before the standard optogenetic stimulation (30s pre-stimulation period followed by 30s 655nm photostimulation) was applied. The preference index was calculated as follows,

$$\text{preference index} = \frac{n_{\text{left}} - n_{\text{right}}}{n_{\text{left}} + n_{\text{right}}},$$

where n_{left} is the number of approach bouts toward the left target and n_{right} is the number of approach bouts toward the right target.

Loss-of-function assay. The inwardly-rectifying potassium channel Kir2.1 was expressed in CAP (Figure 3C), MAP (Figures 5B-C), fpC1 (Figures 6D-E), Tk (Figure S7A), and Eb5 (Figure S7B) neurons to test how silencing these neurons affects naturally- or artificially-induced aggression. Experimental flies were single-housed for six days under a normal 12hr-light: 12hr-dark cycle to naturally enhance aggressiveness (Figures 3C, 5B, and 6D). For behavioral epistasis experiments where fighting was optogenetically induced experimental flies were group-housed and reared in dark (Figures 5C, 6E, and S7).

Optogenetic activation in isolated flies experiment (Figures 7B-C). GH>GH flies and GH>SH testers were collected on the same day. Both fly groups were reared in the GH condition for the first three days. On the fourth day, the control (GH>GH) flies were transferred to a new vial and maintained in groups whereas the testers (GH>SH) were isolated individually; both groups were maintained for an additional 3 days. The optogenetic stimulation experiments were done on the morning of the 7th day. Here we purposely shortened the length of social isolation from six days (our standard procedure; Wang et al., 2008) to three days, in order to avoid a ceiling effect on aggression.

Functional imaging

Six-day old experimental flies were briefly anesthetized on ice and head-fixed on a customized holder with the UV glue in their normal standing posture. The top of the fly head was immersed in fly saline (103mM NaCl, 3mM KCl, 5mM N-Tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid, 8mM trehalose, 10mM glucose, 26mM $NaHCO_3$, 1mM $NaH2PO_4$, 4mM MgCl2, 1.5mM CaCl2; pH7.25; 270-275mOsm) (Hong and Wilson, 2015). A piece of cuticle (~350 μm by 350 μm) was removed from the posterior side of the head capsule to create an imaging window. After surgery, the experimental fly was placed under a 0.8 numerical aperture (NA) 40x objective (LUMPLFLN40XW, Olympus) and habituated for at least 5 minutes. The optical setup for two-photon imaging with optogenetic activation was as described in Inagaki et al., 2014. Briefly, imaging was performed using a custom-modified Ultima two-photon laser scanning microscope (Bruker). 920nm ultrafast light pulses for exciting GCaMP was provided by a Chameleon Ultra II Ti:Sapphire laser (Coherent) and the GCaMP signals were detected by photomultiplier-tubes (Hamamatsu). Images were acquired at 256x256 pixel resolution and 2 frames per second. Chrimson activation was provided by a fiber-coupled 660nm LED (M660F1, Thorlabs), powered by a 1-channel LED driver with pulse modulation (DC2100, Thorlabs) and delivered through an optical fiber (200μm in diameter, 0.39NA, M75L01, Thorlabs) placed above the imaging window at a ~45º angle and ~500μm in distance. The stimulation paradigm included a 20s

baseline, 5s photo-stimulation at the designated frequency (5-50Hz) and intensity (0-34.6 μW), and a 35s post-stimulation period.

Labeling neurons with photoactivatable GFP

To trace the morphologies of different Eb5 cell classes, photoactivatable-GFP (PA-GFP; Datta et al., 2008; Ruta et al., 2010) was expressed by Eb5-Gal4 (attp2) in both sexes. The experimental preparation was similar to functional imaging experiments except that the photoactivation was provided by 710 nm ultrafast light pulses using the two-photon microscope. The photoactivation was first localized to the cell body of the targeted neuron and, after the diffusion of the activated PA-GFP, applied subsequently to the terminus of the labeled segments to extend the labeling. Photoactivation cycles were separated by 10min intervals to allow the spread of activated PA-GFP and the entire experiment typically lasted 1.5-2 hours for each cell. Image stacks were taken at 1024x1024 pixel resolution. For the purpose of clarity, the labeled neuron was traced with Simple Neurite Tracer plugins using Fiji to mask the background fluorescence. The morphology of each cell class was confirmed with at least two biological replicates.

Statistics and quantification

Statistical analysis was performed using MATLAB. All behavioral data were compared using nonparametric tests. The n number for each experiment is indicated in the figures and listed in Table S3, along with the statistical method used for each comparison, the *p*-value, and the test scores. Briefly, Mann-Whitney U-tests and Wilcoxon signed-rank tests were used for between and within-group comparisons, respectively. The cutoff for significance was set as an □<0.05. The central mark of each boxplot indicates the median and the bottom and top are the 25th and the 75th percentiles, respectively.

Fly tracking and behavior classification

Behavioral videos were tracked with Caltech FlyTracker to determine the positions, the velocities, and the postures of the experimental flies in 30 fps recordings (Eyjolfsdottir et al.,

2014). These tracking data were used by behavioral classifiers developed with the Janelia Automatic Animal Behavior Annotator (JAABA; Kabra et al., 2013) to determine the behavioral bouts. Naturally occurring fighting between single-housed male-male or female-female wild-type pairs were used to train the classifiers. We used the following criteria to train the classifiers for approach, lunge, and headbutt, separately: An approach bout starts when the two flies are at a distance (at least one fly-body length(~3mm) apart). The behaving fly first orients toward the target fly, moves forward with acceleration, and finally makes contact with the target (e.g., their legs are crossing or their bodies touching). We used the same approach classifier for detecting male and female approach bouts, and classifier performance was comparable between the two sexes (Figure S1D). We considered lunging and headbutting as short-distance behaviors. The behaving fly only lunges or headbutts when the target fly is in close proximity. Also, lunging and headbutting are very brief actions whereas the duration of approach may vary depending on the distance and the velocity of the behaving fly. A lunge bout begins when the behaving fly raises its front legs and its upper body and "slams down" onto the target fly. We consider lunging as a male-specific behavior because it was only observed during male fights in our experiments; application of the automated lunge classifier to videos of female fights yielded only false positives. A headbutt bout was scored when the behaving fly lashed out in a thrust with its head, towards the target. The classifier performance is listed in Table S2. Behavioral bouts annotated by the classifiers were manually curated to eliminate false-positives and false-negatives in all experiments.

Imaging data analysis

Same-size region of interests (ROIs) were manually drawn over the cell bodies of targeted neurons and the GCaMP fluorescence in the ROIs was measured using the ROI manager in Fiji software. The mean of the fluorescence intensity during the first 20s of image acquisition was used as the baseline fluorescence (F) to calculate the $\Delta F/F$. The $\Delta F/F$ was then normalized to the maximum peak value of fluorescence detected within the full data set that combined data from all genotypes involved, and is presented as $\Delta F/F$ (%). The mean of $\Delta F/F$ during the indicated periods (5s before, during, and after photo-stimulation) was calculated

and compared using the Wilcoxon signed-rank (WSR) test and Mann-Whitney U (MWU) test in MATLAB. Specifically, MWU was used for between-genotype comparison whereas WSR was used for within-genotype pre-stimulation (Pre-stim) versus during stimulation (Stim) comparisons. The cutoff for significance was set as $\alpha<0.05$. Each data point represents one cell per fly. For each genotype, the testing flies were collected from two independent crosses to avoid sampling bias.

References

Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., Amanatides, P.G., Scherer, S.E., Li, P.W., Hoskins, R.A., Galle, R.F., et al. (2000). The genome sequence of *Drosophila melanogaster*. Science *287*, 2185–2195.

Anderson, D.J. (2016). Circuit modules linking internal states and social behaviour in flies and mice. Nat. Rev. Neurosci. 17, 692-704.

Anderson, D.J., and Adolphs, R. (2015). A framework for studying emotions across phylogeny. Cell *157*, 187-200.

Asahina, K., Watanabe, K., Duistermars, B.J., Hoopfer, E., Gonzalez, C.R., Eyjolfsdottir, E.A., Perona, P., and Anderson, D.J. (2014). Tachykinin-expressing neurons control male-specific aggressive arousal in *Drosophila*. Cell *156*, 221-235.

Asahina, K. (2018). Sex differences in *Drosophila* behavior: qualitative and quantitative dimorphism. Curr. Opin. Physiol. *6*, 35–45.

Baines, R.A., Uhler, J.P., Thompson, A., Sweeney, S.T., and Bate, M. (2001). Altered electrical properties in *Drosophila* neurons developing without synaptic transmission. J. Neurosci. *21*, 1523-1531.

Bennet-Clark, H.C., and Ewing, A.W. (1967). Stimuli provided by courtship of male *Drosophila* melanogaster. Nature *215*, 669–671.

Blanchard, R.J., Fukunaga, K., Blanchard, D.C., and Kelley, M.J. (1975). Conspecific aggression in the laboratory rat. Comp. Physiol. Psychol. *89*, 1204–1209.

Cachero, S., Ostrovsky, A.D., Yu, J.Y., Dickson, B.J., and Jefferis, G.S. (2010). Sexual dimorphism in the fly brain. Curr. Biol. *20*, 1589-1601.

Chan, Y.-B., and Kravitz, E.A. (2007). Specific subgroups of FruM neurons control sexually dimorphic patterns of aggression in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA *104*, 19577-19582.

Chavez, A., Tuttle, M., Pruitt, B.W., Ewen-Campen, B., Chari, R., Ter-Ovanesyan, D., Haque, S.J., Cecchi, R.J., Kowal, E.J.K., Buchthal, J., et al. (2016). Comparison of Cas9 activators in multiple species. Nat. Methods *13*, 563–567.

Chen, S., Lee, A.Y., Bowens, N.M., Huber, R., and Kravitz, E.A. (2002). Fighting fruit flies: a model system for the study of aggression. Proc. Natl. Acad. Sci. USA *99*, 5664-5668.

Chiang, A.S., Lin, C.Y., Chuang, C.C., Chang, H.M., Hsieh, C.H., Yeh, C.W., Shih, C.T., Wu, J.J., Wang, G.T., Chen, Y.C., et al. (2011). Three-dimensional reconstruction of brain-wide wiring networks in *Drosophila* at single-cell resolution. Curr. Biol. *21*, 1–11.

Chiara, V., Ramon Portugal, F., and Jeanson, R. (2019). Social intolerance is a consequence, not a cause, of dispersal in spiders. PLoS Biol. *17*, e3000319.

Costa, M., Manton, J.D., Ostrovsky, A.D., Prohaska, S., and Jefferis, G.S. (2016). NBLAST: rapid, sensitive comparison of neuronal structure and construction of neuron family databases. Neuron *91*, 293-311.

Craig, W. (1917). Appetites and aversions as constituents of instincts. Bio. Bulletin *34*, 91-107.

Dana, H., Sun, Y., Mohar, B., Hulse, B.K., Kerlin, A.M., Hasseman, J.P., Tsegaye, G., Tsang, A., Wong, A., Patel, R., et al. (2019). High-performance calcium sensors for imaging activity in neuronal populations and microcompartments. Nat. Methods *16*, 649–657.

Datta, S.R., Vasconcelos, M.L., Ruta, V., Luo, S., Wong, A., Demir, E., Flores, J., Balonze, K., Dickson, B.J., and Axel, R. (2008). The *Drosophila* pheromone cVA activates a sexually dimorphic neural circuit. Nature *452*, 473-477.

Deutsch, D., Pacheco, D.A., Encarnacion-rivera, L., Pereira, T., Fathy, R., Ireland, E.C., Burke, A.T., Dorkenwald, S., McKellar, C., Macrina, T.*, et al.* (2020). The neural basis for a persistent internal state in *Drosophila* females. bioRxiv. DOI: 10.1101/2020.02.13.947952.

Duistermars, B.J., Pfeiffer, B.D., Hoopfer, E.D., Anderson, D.J., Duistermars, B.J., Pfeiffer, B.D., Hoopfer, E.D., and Anderson, D.J. (2018). A brain module for scalable control of complex multi-motor threat displays. Neuron *100*, 1474–1490.

Dulac, C., and Kimchi, T. (2007). Neural mechanisms underlying sex-specific behaviors in vertebrates. Curr. Opin. Neurobiol. *17*, 675–683.

Eyjolfsdottir, E., Branson, S., Burgos-Artizzu, X.P., Hoopfer, E.D., Schor, J., Anderson, D.J., and Perona, P. (2014). Detecting social actions of fruit flies. Lect. Notes Comput. Sci. *8690*, 772–787.

Falkner, A.L., Dollar, P., Perona, P., Anderson, D.J., and Lin, D. (2014). Decoding ventromedial hypothalamic neural activity during male mouse aggression. J. Neurosci. *34*, 5971-5984.

Falkner, A.L., Grosenick, L.D., Thomas J., Deisseroth, K., and Lin, D. (2016). Hypothalamic control of male aggression-seeking behavior. Nat. Neurosci. *19*, 596-604.

Fernández, M.P., Chan, Y.B., Yew, J.Y., Billeter, J.C., Dreisewerd, K., Levine, J.D., and Kravitz, E.A. (2010). Pheromonal and behavioral cues trigger male-to-female aggression in *Drosophila*. PLoS Bio. *8*, e1000541.

Gao, X.J., Riabinina, O., Li, J., Potter, C.J., Clandinin, T.R., and Luo, L. (2015). A transcriptional reporter of intracellular Ca^{2+} in *Drosophila*. Nat. Methods *18*, 917–925.

Gentry, R.N., Schuweiler, D.R., and Roesch, M.R. (2019). Dopamine signals related to appetitive and aversive events in paradigms that manipulate reward and avoidability. Brain Res. *15*, 80-90.

Hashikawa, K., Hashikawa, Y., Falkner, A., and Lin, D. (2016). The neural circuits of mating and fighting in male mice. Curr. Opin. Neurobiol. *38*, 27–37.

Hashikawa, K., Hashikawa, Y., Tremblay, R., Zhang, J., Feng, J.E., Sabol, A., Piper, W.T., Lee, H., Rudy, B., and Lin, D. (2017). Esr1+cells in the ventromedial hypothalamus control female aggression. Nat. Neurosci. *20*, 1580-1590.

Hobert, O., and Kratsios, P. (2019). Neuronal identity control by terminal selectors in worms, flies, and chordates. Curr. Opin. Neurobiol. *56*, 97-105.

Hong, E.J., and Wilson, R.I. (2015). Simultaneous encoding of odors by channels with diverse sensitivity to inhibition. Neuron *85*, 573–589.

Hoopfer, E.D. (2016). Neural control of aggression in *Drosophila*. Curr. Opin. Neurobiol. *38*, 109-118.

Hoopfer, E.D., Jung, Y., Inagaki, H.K., Rubin, G.M., and Anderson, D.J. (2015). P1 interneurons promote a persistent internal state that enhances inter-male aggression in *Drosophila*. Elife *4*, e11346.

Hoyer, S.C., Eckart, A., Herrel, A., Zars, T., Fischer, S.A., Hardie, S.L., and Heisenberg, M. (2008). Octopamine in male aggression of *Drosophila*. Curr. Biol. *18*, 159-167.

Inagaki, H.K., Jung, Y., Hoopfer, E.D., Wong, A.M., Mishra, N., Lin, J.Y., Tsien, R.Y., and Anderson, D.J. (2014). Optogenetic control of freely behaving adult *Drosophila* using a red-shifted channelrhodopsin. Nat. Methods *11*, 325–332.

Ishii, K., Wohl, M., Desouza, A., Asahina, K., Diego, S., and States, U. (2020). Sex-determining genes distinctly regulate courtship capability and target preference via sexually dimorphic neurons, Elife *9*, e52701.

Jenett, A., Rubin, G.M., Ngo, T.-T.B., Shepherd, D., Murphy, C., Dionne, H., Pfeiffer, B.D., Cavallaro, A., Hall, D., Jeter, J., et al. (2012). A GAL4-driver line resource for *Drosophila* neurobiology. Cell Rep. *2*, 991–1001.

Jennings, J.H., Ung, R.L., Resendez, S.L., Stamatakis, A.M., Taylor, J.G., Huang, J., Veleta, K., Kantak, P.A., Aita, M., Shilling-Scrivo, K*., et al.* (2015). Visualizing hypothalamic network dynamics for appetitive and consummatory behaviors. Cell *160*, 516-527.

Jung, Y., Kennedy, A., Chiu, H., Mohammad, F., Claridge-Chang, A., and Anderson, D.J. (2020). Neurons that function within an integrator to promote a persistent behavioral state in *Drosophila*. Neuron *105*, 1–12.

Kabra, M., Robie, A.A., Rivera-Alba, M., Branson, S., and Branson, K. (2013). JAABA: Interactive machine learning for automatic annotation of animal behavior. Nat. Methods *10*, 64–67.

Kim, D.W., Yao, Z., Graybuck, L.T., Nguyen, T.N., Smith, K.A., Fong, O., Yi, L., Koulena, N., Pierson, N., Shah, S*., et al.* (2019). Multimodal analysis of cell types in a hypothalamic node controlling social behavior. Cell *179*, 713-728.

Kimura, K., Hachiya, T., Koganezawa, M., Tazawa, T., and Yamamoto, D. (2008). Fruitless and doublesex coordinate to generate male-specific neurons that can initiate courtship. Neuron *59*, 759–769.

Klapoetke, N.C., Murata, Y., Kim, S.S., Pulver, S.R., Birdsey-Benson, A., Cho, Y.K., Morimoto, T.K., Chuong, A.S., Carpenter, E.J., Tian, Z*., et al.* (2014). Independent optical excitation of distinct neural populations. Nat. Methods *11*, 338-346.

Koganezawa, M., Kimura, K.i., and Yamamoto, D. (2016). The neural circuitry that functions as a switch for courtship versus aggression in *Drosophila* males. Curr. Biol. *26*, 1395-1403.

Kravitz, E.A., and Fernández, M.P. (2015). Aggression in *Drosophila*. Beh. Neurosci. *129*, 549-563.

Lee, H., Kim, D.W., Remedios, R., Anthony, T.E., Chang, A., Madisen, L., Zeng, H., and Anderson, D.J. (2014). Scalable control of mounting and attack by Esr1+ neurons in the ventromedial hypothalamus. Nature *509*, 627-632.

Lim, R.S., Eyjolfsdottir, E., Shin, E., Perona, P., and Anderson, D.J. (2014). How food controls aggression in *Drosophila*. Plos One *9*, e105626.

Longair, M.H., Baker, D.A., and Armstrong, J.D. (2011). Simple neurite tracer: Open source software for reconstruction, visualization and analysis of neuronal processes. Bioinformatics *27*, 2453–2454.

Lorenz, K. (1950). The comparative method in studying innate behavior patterns. In Physiological Mechanisms in Animal Behavior. Society's Symposium IV (Oxford: Academic Press), 221–268.

Luo, L., Callaway, E.M., and Svoboda, K. (2008). Genetic dissection of neural circuits. Neuron *57*, 634-660.

Manoli, D.S., Fan, P., Fraser, E.J., and Shah, N.M. (2013). Neural control of sexually dimorphic behaviors. Curr. Opin. Neurobiol. *23*, 330–338.

Masse, N.Y., Cachero, S., Ostrovsky, A.D., and Jefferis, G.S. (2012). A mutual information approach to automate identification of neuronal clusters in *Drosophila* brain images. Front. Neuroinform. *6*, 21.

McKellar, C.E., Lillvis, J.L., Bath, D.E., Fitzgerald, J.E., Cannon, J.G., Simpson, J.H., Dickson, B.J., Mckellar, C.E., Lillvis, J.L., Bath, D.E., et al. (2019). Threshold-based ordering of sequential actions during *Drosophila* courtship. Curr. Biol. *29*, 426–434.

Mohammad, F., Stewart, J.C., Ott, S., Chlebikova, K., Chua, J.Y., Koh, T.W., Ho, J., and Claridge-Chang, A. (2017). Optogenetic inhibition of behavior with anion channelrhodopsins. Nat. Methods *14*, 271–274.

Nilsen, S.P., Chan, Y.-B., Huber, R., and Kravitz, E.A. (2004). Gender-selective patterns of aggressive behavior in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA *101*, 12342-12347.

Palavicino-Maggio, C.B., Chan, Y.B., McKellar, C., and Kravitz, E.A. (2019). A small number of cholinergic neurons mediate hyperaggression in female *Drosophila*. Proc. Natl. Acad. Sci. USA *116*, 17029-17038.

Pavlou, H.J., Lin, A.C., Neville, M.C., Nojima, T., Diao, F., Chen, B.E., White, B.H., and Goodwin, S.F. (2016). Neural circuitry coordinating male copulation. ELife *5*, e20713.

Pfeiffer, B.D., Jenett, A., Hammonds, A.S., Ngo, T.T.B., Misra, S., Murphy, C., Scully, A., Carlson, J.W., Wan, K.H., Laverty, T.R., et al. (2008). Tools for neuroanatomy and neurogenetics in *Drosophila*. Proc. Natl. Acad. Sci. USA *105*, 9715–9720.

Pfeiffer, B.D., Ngo, T.-T.B., Hibbard, K.L., Murphy, C., Jenett, A., Truman, J.W., and Rubin, G.M. (2010). Refinement of tools for targeted gene expression in *Drosophila*. Genetics *186*, 735–755.

Remedios, R., Kennedy, A., Zelikowsky, M., Grewe, B.F., Schnitzer, M.J., and Anderson, D.J. (2017). Social behaviour shapes hypothalamic neural ensemble representations of conspecific sex. Nature *550*, 388-392.

Rezával, C., Nojima, T., Neville, M.C., Lin, A.C., and Goodwin, S.F. (2014). Sexually dimorphic octopaminergic neurons modulate female postmating behaviors in *Drosophila*. Curr. Biol. *24*, 725–730.

Rideout, E.J., Dornan, A.J., Neville, M.C., Eadie, S., and Goodwin, S.F. (2010). Control of sexual differentiation and behavior by the *doublesex* gene in *Drosophila melanogaster*. Nat. Neurosci. *13*, 458-467.

Ruta, V., Datta, S.R., Vasconcelos, M.L., Freeland, J., Looger, L.L., and Axel, R. (2010). A dimorphic pheromone circuit in *Drosophila* from sensory input to descending output. Nature *468*, 686-690.

Salamone, J.D., and Correa, M. (2012). The mysterious motivational functions of mesolimbic dopamine. Neuron *76*, 470-485.

Schretter, C.E., Aso, Y., Robie, A.A., Dreher, M., Dolan, M.-J., Chen, N., Ito, M., Yang, T., Parekh, R., Branson, K.M*., et al.* (2020). Cell types and neuronal circuitry underlying female aggression in *Drosophila*. eLife, e58942.

Shelly, T.E. (1999). Defense of oviposition sites by female oriental fruit flies (Diptera: Tephritidae). Florida Entomol. *82*, 339-346.

Sgoifo, A., Stilli, D., Musso, E., Mainardi, D., and Parmigiani, S. (1992). Offensive and defensive bite-target topographies in attacks by lactating rats. Aggress. Behav. *21*, 79–89.

Stockinger, P., Kvitsiani, D., Rotkopf, S., Tirián, L., and Dickson, B.J. (2005). *Drosophila* Male Courtship Behavior. Cell *121*, 795–807.

Tinbergen, N. (1951). The study of instinct (Clarendon Press/Oxford University Press).

Toth, M., Halasz, J., Mikics, E., Barsy, B., and Haller, J. (2008). Early social deprivation induces disturbed social communication and violent aggression in adulthood. Behav. Neurosci. *122*, 849-854.

Ueda, A., and Kidokoro, Y. (2002). Aggressive behaviours of female *Drosophila melanogaster* are influenced by their social experience and food resources. Physiol. Entomol. *27*, 21-28.

von Philipsborn, A.C., Liu, T., Yu, J.Y., Masser, C., Bidaye, S.S., and Dickson, B.J. (2011). Neuronal control of *Drosophila* courtship song. Neuron *69*, 509-522.

Vrontou, E., Nilsen, S.P., Demir, E., Kravitz, E.A., and Dickson, B.J. (2006). *fruitless* regulates aggression and dominance in *Drosophila*. Nat. Neurosci. *9*, 1469-1471.

Wang, F., Wang, K., Forknall, N., Patrick, C., Yang, T., Parekh, R., Bock, D., and Dickson, B.J. (2020). Neural circuitry linking mating and egg laying in *Drosophila* females. Nature *579*, 101-105.

Wang, L., Dankert, H., Perona, P., and Anderson, D.J. (2008). A common genetic target for environmental and heritable influences on aggressiveness in *Drosophila*. Proc. Natl. Acad. Sci. USA *105*, 5657-5663.

Watanabe, K., Chiu, H., Pfeiffer, B.D., Wong, A.M., Hoopfer, E.D., Rubin, G.M., and Anderson, D.J. (2017). A circuit node that integrates convergent input from neuromodulatory and social behavior-promoting neurons to control aggression in *Drosophila*. Neuron *95*, 1112-1128.

Yamamoto, D., and Koganezawa, M. (2013). Genes and circuits of courtship behaviour in *Drosophila* males. Nat. Rev. Neurosci. *14*, 681-692.

Yang, C.F., Chiang, M.C., Gray, D.C., Prabhakaran, M., Alvarado, M., Juntti, S.A., Unger, E.K., Wells, J.A., and Shah, N.M. (2013). Sexually dimorphic neurons in the ventromedial hypothalamus govern mating in both sexes and aggression in males. Cell *153*, 896-909.

Yang, C.F., and Shah, N.M. (2014). Representing sex in the brain, one module at a time. Neuron *82*, 261-278.

Yu, J.Y., Kanai, M.I., Demir, E., Jefferis, G.S., and Dickson, B.J. (2010). Cellular organization of the neural circuit that drives *Drosophila* courtship behavior. Curr. Biol. *20*, 1602-1614.

Zelikowsky, M., Hui, M., Karigo, T., Choe, A., Yang, B., Blanco, M.R., Beadle, K., Gradinaru, V., Deverman, B.E., and Anderson, D.J. (2018). The neuropeptide Tac2 controls a distributed brain state induced by chronic social isolation stress. Cell *173*, 1265-1279.

Chapter 3

FUTURE DIRECTIONS

In this chapter, I would like to discuss the unsolved questions and my perspectives on the future research. These questions are divided into two categories: the follow-up questions after the project presented in the second chapter, and the all-around neuroscience inquiries. In the end, I will also point out what methods are critically needed for accelerating *Drosophila* neuroscience research.

Functional connectivity between the modules of aggression circuits

Several questions have emerged from the discovery of the functional connectivity between CAP and MAP/ fpC1 neurons in the two sexes of *Drosophila*. A major question is, how are CAP, MAP, and fpC1 neurons related to other aggression-promoting neurons. This question can also be interpreted as, how do neural modules coordinate with each other to regulate aggression.

The approach for addressing this question, in my opinion, should be different in males versus females because our understanding of the circuits that govern male versus female aggression is different. At the sensory input level, it has been shown in males that the cuticular hydrocarbons 11-cis-vaccenyl acetate and (z)-7-tricosene promote intermale aggression through the detection by the O67d+ olfactory receptor neurons and Gr32a-expressing gustatory neurons, respectively (Wang and Anderson, 2010; Wang et al., 2011). Deeper in the circuit, a group of male-specific P1a neurons is shown to promote social arousal (Hoopfer et al., 2015). There are also neurons that control specific aggressive actions, such as the threat neurons (Duistermars et al., 2018). Although the downstream descending neurons and motor circuits remain unknown, many neuromodulatory modules have been identified, including Tachykinin (Tk)-, Octopamine- and its receptor, Serotonin-, and Dopamine-expressing neurons (Alekseyenko et al., 2014; Andrews et al., 2014; Asahina et al., 2014; Hoyer et al., 2008; Watanabe et al., 2017). Therefore, it is important to consider the interactions between

the neuromodulatory system and the aggression circuit when discussing the relationships between CAP, MAN, and the other identified circuit modules.

We showed that Tk neurons likely act downstream of CAP and MAP neurons to promote intermale aggression (Chiu et al., 2021). However, Tk neurons also act downstream of the P1a neurons (Hoopfer et al., 2015). It thus suggests two possible circuit organizations, one is that CAP, MAP, P1a, and Tk neurons reside in the same circuit whereas the other is that CAP, MAP, and P1a act in parallel to activate Tk neurons. To verify these two possibilities, we first have to determine the relationship between CAP, MAP, and P1a neurons. Specifically, MAP and P1a are both considered as subsets of pC1 neurons (Asahina, 2018; Chiu et al., 2021; Hoopfer, 2016; Hoopfer et al., 2015; Koganezawa et al., 2016). How information is transformed between these pC1 subclasses will be critical for the comparison between the male and female aggression circuits. How they act on Tk neurons to elicit neuromodulation on aggression also remains to be elucidated. Interestingly, these Tk neurons present only in males (Asahina et al., 2014), which suggests that sexual differences in fly aggression can be partially attributed to the sexually dimorphic neuromodulation mediated by Tachykinin.

Much less is known about the female aggression circuits. Two groups of neurons, aIPg and pC1d, have been recently shown to promote female aggression (Deutsch et al., 2020; Palavicino-Maggio et al., 2019; Schretter et al., 2020). Because the fpC1 driver labels more than one subclass of pC1 neurons, an immediate question is whether the aggression phenotype induced by fpC1 activation is mediated by pC1d neurons (Chiu et al., 2021; Deutsch et al., 2020; Schretter et al., 2020). We unfortunately cannot resolve this question by directly comparing the driver expressions because of the driver compatibility issue. A possible solution is to make a split-LexA driver for labeling fpC1 or pC1d neurons (Gao et al., 2015; Ting et al., 2011). Alternatively, we can also try to identify fpC1 neurons in the female connectomic database and compare them with pC1d (Dorkenwald et al., 2020; Scheffer et al., 2020; Zheng et al., 2018). It requires brains that contain individually PA-

GFP-traced fpC1 neurons to be precisely registered to the standard brain template, for which the process is in progress.

Nevertheless, two interesting discoveries from the studies of aIPg and pC1d neurons point out important directions for future research. Firstly, the reciprocal connections between aIPg and pC1d neurons revealed by EM connectome provide a structure-based hypothesis for how persistent behavior is generated (Deutsch et al., 2020; Schretter et al., 2020). To test this possibility, we should first determine which classes of neurons carry the persistent activity that is correlated with the prolonged behavioral responses. In other words, when activating one of the neuronal class, whether the persistent neuronal activity is induced in itself or the other. We can then test if such persistent activity is interrupted if any of the two neuronal classes is suppressed.

The second important observation is that different female pC1 subclasses are involved in distinct behaviors (Deutsch et al., 2020; Schretter et al., 2020; Wang et al., 2020, 2021). According to EM connectomic tracing, these cholinergic pC1 subclasses are heavily interconnected, and some even share the same downstream targets (Deutsch et al., 2020; Schretter et al., 2020; Wang et al., 2020, 2021). This poses an intriguing paradox: why does the activation of a specific pC1 subclass elicit one behavior exclusively? *Drosophila* is at an advantageous position for solving such a paradox because the release of the EM traced connectivity provides a physical map for tracing possible information flow in these intertwined circuits. Moreover, the advanced genetic and molecular toolkits allow us to monitor and manipulate activity of specific neuronal populations (Simpson, 2009). The combination of these two advantages enables us to generate an activity matrix that documents the signal transduction between individual pC1 subclasses and their downstream targets. This activity matrix can then be referenced when examining the behavioral outputs, which would ultimately piece out how inputs are processed between these closely related circuits and transformed into specific behaviors. Importantly, such observation seems to be applicable to males although the cell type classification is simply based on the expression of *doublesex* and *fruitless* alone at this point (Koganezawa et al., 2016). Once the male connectomic

database and more drivers for specific labelling of male pC1 subclasses become available, the comparison between the male versus female activity matrices of the pC1 cluster would be very informative for understanding how sexual differences in behaviors are implemented at the circuit level.

Neural mechanisms underlying behavioral plasticity

As discussed in the first chapter, males and females adapt differently to various extrinsic and intrinsic triggers of aggression. One of the common triggers that enhances aggression of both sexes is social isolation (Chiu et al., 2021; Ueda and Kidokoro, 2002; Wang et al., 2008). We showed in the study presented in the second chapter that the enhanced aggressiveness is resulted partially from the strengthened connectivity between CAP and MAP/fpC1 neurons (Chiu et al., 2021). Also, social isolation does not change the excitability in either CAP or MAP/fpC1 neurons themselves (Chiu et al., 2021). Therefore, depending on whether the connection is direct or indirect, the strengthening effect may be caused by changes that occur at the synapses of theses neurons or in the cells that connect CAP and MAP/fpC1 neurons, respectively. Additionally, such changes may also occur in other places along the aggression circuit. Thus, it is critical to determine if the causality is mediated by changes at the synaptic or at the cellular level as well as if the connectivity between CAP and MAP/fpC1 neurons is exclusively strengthened after social isolation.

No matter where in the circuit these structural changes occur, corresponding modification in gene expression profile is expected. Global survey of isolation-dependent gene expression in either the head tissue or the nervous system yields some promising candidates whose expression suppresses aggression among group-housed males (Wang et al., 2008) or both sexes (Ishii et al., 2020). However, we are still far from the complete understanding of the gene regulation that is causally related to the isolation-dependent increase in aggression. Perhaps documenting the dynamics of the transcriptomics on a more fine-grained time course, for example, every two days of isolation as compared to only once after the 6-day isolation, as well as performing a more circuit-focused gene expression profiling, such as

conducting single-cell sequencing on the *doublesex*-expressing neurons in both sexes, would help to resolve any transient or subtle changes that are masked by the global approaches.

It is important to also note that aggression is not the only social behavior affected by isolation. Male-to-female courtship is greatly enhanced by isolation as well (Dankert et al., 2009). Contrary to what has been observed in the aggression circuit, obvious changes in the excitability of the courtship circuit module, P1a neurons, have been documented in isolated males (Inagaki et al., 2014). It thus suggests that different mechanisms are adopted to modulate the circuit activity of different social behaviors during isolation. Comparing and contrasting these circuit-based differences would provide additional insights into the neural basis of behavioral plasticity.

Mating experience is another factor that alter aggressiveness of both sexes (Bath et al., 2017; Baxter et al., 2015; Yuan et al., 2014). Two observations are worth considering for future investigations. Firstly, female aggression is stimulated by the transfer of sperm or sex peptide after mating (Bath et al., 2017). It suggests that the causality for increased aggression between mated males is different from the one for females. Secondly, unlike social isolation, the effect of mating experience on aggression is irreversible (Wang et al., 2008), which means that the changes in the organization of aggression circuits induced by mating experience are permanent. To investigate where and how such permanent changes are established may require our further understanding of the interactions between courtship and aggression circuits in both sexes.

Tools for advancing neuroscience research in *Drosophila*

Drosophila has been used intensively for studying the neural basis of behaviors for more than a decade (Olsen and Wilson, 2008; Simpson, 2009). The combination of specific labeling lines as well as versatile effectors for monitoring and manipulating neuronal function enables researchers to systematically identify the neurons involved in specific behaviors, map the functional connectivity between these neurons, and finally define the

causal relationships between the circuit activity and behaviors in flies. Recently, the release of EM connectomic database further facilitates the formation of structure-based hypotheses, which can speed up the process of circuit mapping immensely (Dorkenwald et al., 2020; Scheffer et al., 2020; Zheng et al., 2018). Despite these advantages, the progress of neuroscience research in *Drosophila* has been hindered by several technical limitations. In this section, I will discuss three critical techniques that are less developed in the field.

The first technique is *in vivo* imaging in freely moving flies. Monitoring the neural activity during behaviors is critical for decoding the neural computation and determining the causality. However, due to the body-size constraint, the cutting-edge techniques that are widely used in rodents, such as miniature head-mounted microscope or multichannel silicon probes, are impracticable in flies (Sariev et al., 2017; Ghosh et al., 2011). To achieve the goal of recording neural activity in behaving flies, experimental setups with various imaging resolutions have been established. It is already possible to perform imaging or electrophysiological recording in a tethered but freely walking fly under the two-photon microscope (Maimon et al., 2010). Despite of being a revolutionary and powerful tool, it is not suitable for social behavior studies because the range of fly-fly interaction allowed on such imaging setup is limited. To minimize the physical restriction on flies, Flyception has been invented to monitor the neural activity in freely moving flies (Grover et al., 2016, 2020). However, the neural activity is measured based on the photons emitted from a small window on the head of the imaged fly. Therefore, whether the fluorescence change obtained during behaviors belongs to neurons of interest solely depends on the specificity of the driver expression. Ultimately, we wish to establish a system for long-term, high-resolution, *in vivo* recording of neural activity in flies that are free to perform any behaviors. Such a system may be possible if the imaging resolution can be greatly improved in a setup similar to Flyception.

Another technique is activity-dependent expression system. To probe the neuronal causality for behavior, it is important to not only identify the active neuronal ensembles during behavior but to also manipulate the activity of the ensembles afterward and test how the corresponding behavior is affected. Therefore, various activity-dependent expression

systems relying on either the changes in calcium concentration or immediate early gene (IEG) expression have been established in rodents (DeNardo and Luo, 2017). Such systems, however, are not easily adapted for fly research. One of the reasons is that the identification of IEGs in flies has not been straightforward (Chen et al., 2016; Sommerlandt et al., 2019). Only until recently *hr38* has been identified as a promising IEG in insects and its promoter activity is shown to successfully label the active neuronal ensemble in male fly brains during courtship behavior (Fujita et al., 2013; Takayanagi-Kiya and Kiya, 2019). A caveat is that *hr38* expression is required during development, which means that its promoter activity does not exclusively reflect neuronal activity (Kalay et al., 2016; Sekine et al., 2011). How to eliminate endogenous *hr38* promoter activity without affecting animal's physiology is a critical criterion for improvement. TRIC, on the other hand, captures neural activity based on changes in the intracellular calcium concentration (Gao et al., 2015). The reconstitution of the split Gal4 driver used in TRIC relies on the calcium-induced binding between calmodulin and its target peptide (Gao et al., 2015). This system works well for capturing the active neural ensemble for long-term behavior, such as sleep. It is, however, underperformed for short-term behavior, because the reporter expression level driven by TRIC is restricted by how long the intracellular calcium concentration remains high during behavior. Perhaps one way to resolve this issue is to establish permanent reporter expression after the transient rise of intracellular calcium concentration. It may be achieved by replacing the split-Gal4 system with split recombinase (Weinberg et al., 2019). Nevertheless, a stable yet sensitive activity-dependent expression system in fly is yet to be established.

The last technique focuses on genetically encoded sensors for various signal transductions in the nervous systems, including the voltage, the neurotransmitter, and the neuropeptide sensors. Although the calcium indicator, GCaMP, is broadly used for monitoring neural activity in both vertebrate and invertebrate studies, it is important to remind ourselves that the GCaMP signals do not necessarily reflect the spike activity of the same cell faithfully, and that they are less suitable for detecting subthreshold activity or hyperpolarization inputs (Lin and Schnitzer, 2016; Theis et al., 2016; Yang et al., 2016). Therefore, the development of voltage sensors would allow us to visualize the neural dynamics with finer details, and to

overcome the challenges of performing electrophysiology in the tiny fly brain. Moreover, the passing of electric potentials is not the only way that neurons communicate. The release of neurotransmitters or neuropeptides are also important readouts for decoding the neural computation in the brain. In fact, the neurotransmitters are also used for the communications between neurons and immune cells (Hodo et al., 2020; Jacobson et al., 2021). Therefore, markers for visualizing the release of these chemical signals and the reactions of their corresponding receptors will benefit both neuroscience-focused and cross-system studies.

References

Alekseyenko, O.V., Chan, Y.-B., Fernandez, M. de la P., Bülow, T., Pankratz, M.J., and Kravitz, E.A. (2014). Single serotonergic neurons that modulate aggression in *Drosophila*. Curr. Biol. *24*, 2700–2707.

Andrews, J.C., Fernández, M.P., Yu, Q., Leary, G.P., Leung, A.K.W., Kavanaugh, M.P., Kravitz, E.A., and Certel, S.J. (2014). Octopamine neuromodulation regulates Gr32a-linked aggression and courtship pathways in *Drosophila* males. PLoS Genet. *10*, e1004356.

Asahina, K. (2018). Sex differences in *Drosophila* behavior: qualitative and quantitative dimorphism. Curr. Opin. Physiol. *6*, 35–45.

Asahina, K., Watanabe, K., Duistermars, B.J., Hoopfer, E., González, C.R., Eyjólfsdóttir, E.A., Perona, P., and Anderson, D.J. (2014). Tachykinin-expressing neurons control male-specific aggressive arousal in *Drosophila*. Cell *156*, 221–235.

AU - Sariev, A., AU - Chung, J., AU - Jung, D., AU - Sharif, F., AU - Lee, J.-Y., AU - Kim, S., and AU - Royer, S. (2017). Implantation of chronic silicon probes and recording of hippocampal place cells in an enriched treadmill apparatus. J. Vis. Exp. e56438.

Bath, E., Bowden, S., Peters, C., Reddy, A., Tobias, J.A., Easton-Calabria, E., Seddon, N., Goodwin, S.F., and Wigby, S. (2017). Sperm and sex peptide stimulate aggression in female *Drosophila*. Nat. Ecol. Evol. *1*, 0154.

Baxter, C.M., Barnett, R., and Dukas, R. (2015). Aggression, mate guarding and fitness in male fruit flies. Anim. Behav. *109*, 235–241.

Chen, X., Rahman, R., Guo, F., and Rosbash, M. (2016). Genome-wide identification of neuronal activity-regulated genes in *Drosophila*. ELife *5*, e19942.

Chiu, H., Hoopfer, E.D., Coughlan, M.L., Pavlou, H.J., Goodwin, S.F., and Anderson, D.J. (2021). A circuit logic for sexually shared and dimorphic aggressive behaviors in *Drosophila*. Cell *184*, 507-520.e16.

Dankert, H., Wang, L., Hoopfer, E.D., Anderson, D.J., and Perona, P. (2009). Automated monitoring and analysis of social behavior in *Drosophila*. Nat. Methods *6*, 297–303.

DeNardo, L., and Luo, L. (2017). Genetic strategies to access activated neurons. Curr. Opin. Neurobiol. *45*, 121–129.

Deutsch, D., Pacheco, D., Encarnacion-Rivera, L., Pereira, T., Fathy, R., Clemens, J., Girardin, C., Calhoun, A., Ireland, E., Burke, A., et al. (2020). The neural basis for a persistent internal state in *Drosophila* females. ELife *9*, e59502.

Dorkenwald, S., McKellar, C., Macrina, T., Kemnitz, N., Lee, K., Lu, R., Wu, J., Popovych, S., Mitchell, E., Nehoran, B., et al. (2020). FlyWire: Online community for whole-brain connectomics (Neuroscience).

Duistermars, B.J., Pfeiffer, B.D., Hoopfer, E.D., and Anderson, D.J. (2018). A brain module for scalable control of complex, multi-motor threat displays. Neuron *100*, 1474-1490.e4.

Fujita, N., Nagata, Y., Nishiuchi, T., Sato, M., Iwami, M., and Kiya, T. (2013). Visualization of neural activity in insect brains using a conserved immediate early gene, Hr38. Curr. Biol. *23*, 2063–2070.

Gao, X.J., Riabinina, O., Li, J., Potter, C.J., Clandinin, T.R., and Luo, L. (2015). A transcriptional reporter of intracellular Ca2+ in *Drosophila*. Nat. Neurosci. *18*, 917–925.

Ghosh, K.K., Burns, L.D., Cocker, E.D., Nimmerjahn, A., Ziv, Y., Gamal, A.E., and Schnitzer, M.J. (2011). Miniaturized integration of a fluorescence microscope. Nat. Methods *8*, 871–878.

Grover, D., Katsuki, T., and Greenspan, R.J. (2016). Flyception: imaging brain activity in freely walking fruit flies. Nat. Methods *13*, 569–572.

Grover, D., Katsuki, T., Li, J., Dawkins, T.J., and Greenspan, R.J. (2020). Imaging brain activity during complex social behaviors in *Drosophila* with Flyception2. Nat. Commun. *11*, 623.

Hodo, T.W., de Aquino, M.T.P., Shimamoto, A., and Shanker, A. (2020). Critical neurotransmitters in the neuroimmune network. Front. Immunol. *11*, 1869.

Hoopfer, E.D. (2016). Neural control of aggression in *Drosophila*. Curr. Opin. Neurobiol. *38*, 109–118.

Hoopfer, E.D., Jung, Y., Inagaki, H.K., Rubin, G.M., and Anderson, D.J. (2015). P1 interneurons promote a persistent internal state that enhances inter-male aggression in *Drosophila*. ELife *4*, e11346.

Hoyer, S.C., Eckart, A., Herrel, A., Zars, T., Fischer, S.A., Hardie, S.L., and Heisenberg, M. (2008). Octopamine in male aggression of *Drosophila*. Curr. Biol. *18*, 159–167.

Inagaki, H.K., Jung, Y., Hoopfer, E.D., Wong, A.M., Mishra, N., Lin, J.Y., Tsien, R.Y., and Anderson, D.J. (2014). Optogenetic control of *Drosophila* using a red-shifted channelrhodopsin reveals experience-dependent influences on courtship. Nat. Methods *11*, 325–332.

Ishii, K., Cortese, M., Leng, X., Shokhirev, M.N., and Asahina, K. (2020). A neurogenetic mechanism of experience-dependent suppression of aggression (Neuroscience).

Jacobson, A., Yang, D., Vella, M., and Chiu, I.M. (2021). The intestinal neuro-immune axis: crosstalk between neurons, immune cells, and microbes. Mucosal Immunol. https://doi.org/10.1038/s41385-020-00368-1

Kalay, G., Lusk, R., Dome, M., Hens, K., Deplancke, B., and Wittkopp, P.J. (2016). Potential direct regulators of the *Drosophila yellow* gene identified by yeast one-hybrid and RNAi screens. G3: Genes, Genomes, Genetics *6*, 3419–3430.

Koganezawa, M., Kimura, K., and Yamamoto, D. (2016). The neural circuitry that functions as a switch for courtship versus aggression in *Drosophila* males. Curr. Biol. *26*, 1395–1403.

Lin, M.Z., and Schnitzer, M.J. (2016). Genetically encoded indicators of neuronal activity. Nat. Neurosci. *19*, 1142–1153.

Maimon, G., Straw, A.D., and Dickinson, M.H. (2010). Active flight increases the gain of visual motion processing in *Drosophila*. Nat. Neurosci. *13*, 393–399.

Olsen, S.R., and Wilson, R.I. (2008). Cracking neural circuits in a tiny brain: new approaches for understanding the neural circuitry of *Drosophila*. Trends Neurosci. *31*, 512–520.

Palavicino-Maggio, C.B., Chan, Y.-B., McKellar, C., and Kravitz, E.A. (2019). A small number of cholinergic neurons mediate hyperaggression in female *Drosophila*. Proc. Natl. Acad. Sci. *116*, 17029–17038.

Scheffer, L.K., Xu, C.S., Januszewski, M., Lu, Z., Takemura, S., Hayworth, K.J., Huang, G.B., Shinomiya, K., Maitlin-Shepard, J., Berg, S., et al. (2020). A connectome and analysis of the adult *Drosophila* central brain. ELife *9*, e57443.

Schretter, C.E., Aso, Y., Robie, A.A., Dreher, M., Dolan, M.-J., Chen, N., Ito, M., Yang, T., Parekh, R., Branson, K.M., et al. (2020). Cell types and neuronal circuitry underlying female aggression in *Drosophila*. ELife *9*, e58942.

Sekine, Y., Takagahara, S., Hatanaka, R., Watanabe, T., Oguchi, H., Noguchi, T., Naguro, I., Kobayashi, K., Tsunoda, M., Funatsu, T., et al. (2011). p38 MAPKs regulate the expression of genes in the dopamine synthesis pathway through phosphorylation of NR4A nuclear receptors. J. Cell Sci. *124*, 3006–3016.

Simpson, J.H. (2009). Chapter 3. Mapping and manipulating neural circuits in the fly brain. In Advances in Genetics, (Elsevier), pp. 79–143.

Sommerlandt, F.M.J., Brockmann, A., Rössler, W., and Spaethe, J. (2019). Immediate early genes in social insects: a tool to identify brain regions involved in complex behaviors and molecular processes underlying neuroplasticity. Cell. Mol. Life Sci. *76*, 637–651.

Takayanagi-Kiya, S., and Kiya, T. (2019). Activity-dependent visualization and control of neural circuits for courtship behavior in the fly *Drosophila melanogaster*. Proc. Natl. Acad. Sci. *116*, 5715–5720.

Theis, L., Berens, P., Froudarakis, E., Reimer, J., Román Rosón, M., Baden, T., Euler, T., Tolias, A.S., and Bethge, M. (2016). Benchmarking spike rate inference in population calcium Imaging. Neuron *90*, 471–482.

Ting, C.-Y., Gu, S., Guttikonda, S., Lin, T.-Y., White, B.H., and Lee, C.-H. (2011). Focusing transgene expression in *Drosophila* by coupling Gal4 with a novel Split-LexA expression system. Genetics *188*, 229–233.

Ueda, A., and Kidokoro, Y. (2002). Aggressive behaviours of female *Drosophila melanogaster* are influenced by their social experience and food resources. Physiol. Entomol. *27*, 21–28.

Wang, L., and Anderson, D.J. (2010). Identification of an aggression-promoting pheromone and its receptor neurons in *Drosophila*. Nature *463*, 227–231.

Wang, F., Wang, K., Forknall, N., Patrick, C., Yang, T., Parekh, R., Bock, D., and Dickson, B.J. (2020). Neural circuitry linking mating and egg laying in *Drosophila* females. Nature *579*, 101–105.

Wang, K., Wang, F., Forknall, N., Yang, T., Patrick, C., Parekh, R., and Dickson, B.J. (2021). Neural circuit mechanisms of sexual receptivity in *Drosophila* females. Nature *589*, 577–581.

Wang, L., Dankert, H., Perona, P., and Anderson, D.J. (2008). A common genetic target for environmental and heritable influences on aggressiveness in *Drosophila*. Proc. Natl. Acad. Sci. *105*, 5657–5663.

Wang, L., Han, X., Mehren, J., Hiroi, M., Billeter, J.-C., Miyamoto, T., Amrein, H., Levine, J.D., and Anderson, D.J. (2011). Hierarchical chemosensory regulation of male-male social interactions in *Drosophila*. Nat. Neurosci. *14*, 757–762.

Watanabe, K., Chiu, H., Pfeiffer, B.D., Wong, A.M., Hoopfer, E.D., Rubin, G.M., and Anderson, D.J. (2017). A circuit node that integrates convergent input from neuromodulatory and social behavior-promoting neurons to control aggression in *Drosophila*. Neuron *95*, 1112-1128.e7.

Weinberg, B.H., Cho, J.H., Agarwal, Y., Pham, N.T.H., Caraballo, L.D., Walkosz, M., Ortega, C., Trexler, M., Tague, N., Law, B., et al. (2019). High-performance chemical- and light-inducible recombinases in mammalian cells and mice. Nat. Commun. *10*, 4845.

Yang, H.H., St-Pierre, F., Sun, X., Ding, X., Lin, M.Z., and Clandinin, T.R. (2016). Subcellular imaging of voltage and calcium signals reveals neural processing *in vivo*. Cell *166*, 245–257.

Yuan, Q., Song, Y., Yang, C.-H., Jan, L.Y., and Jan, Y.N. (2014). Female contact modulates male aggression via a sexually dimorphic GABAergic circuit in *Drosophila*. Nat. Neurosci. *17*, 81–88.

Zheng, Z., Lauritzen, J.S., Perlman, E., Robinson, C.G., Nichols, M., Milkie, D., Torrens, O., Price, J., Fisher, C.B., Sharifi, N., et al. (2018). A complete electron microscopy volume of the brain of adult *Drosophila melanogaster*. Cell *174*, 730-743.e22.