The Role of the *Drosophila* Dopamine 2-Like Receptor in the Blood-Brain Barrier for Male Courtship Behavior

by
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A dissertation submitted to the Department of Biology and Biochemistry,
University of Houston
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in Biology

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May 2020

ACKNOWLEDGMENTS

The journey that brought me to the completion of this dissertation through my research and writings is due in large part to the mentorship and assistance I received in addition to the influences and personal relationships that I developed over the course of the project.

My biggest thanks and gratitude goes out to my Ph.D. advisor and mentor Dr. Brigitte Dauwalder. Dr. Dauwalder provided the framework for doing the experiments that allowed me to complete the research project. In addition to providing the tools, techniques, and research questions that allowed me to perform the experiments, I received excellent mentoring from Dr. Dauwalder in a pleasant workspace and supportive environment that helped tremendously along the way, and I will always be grateful for that.

The assistance I received through various sources during my graduate school experience was a tremendous help and include excellent guidance from the members of my dissertation committee, funding and support from BoBI (Biology of Behavior Institute), help in the lab from UH undergraduate volunteers and students from The Kinkaid School. Fellow graduate students, departmental staff, and friends of mine also helped at various times and in various capacities during the course of this project. Thank you so much to all of you that provided assistance to me, and especially to my committee members: Dr. Richard Bond, Dr. Erin Kelleher, Dr. Arne Lekven and Dr. Gregg Roman.

Last of all, I would like to thank my family, friends, girlfriend and role models that inspire me to follow my dreams, accomplish my goals, and helped to keep me relatively sane and happy through the course of my graduate studies. Many thanks to my stepdad Steve Kopelman for lending a critical ear to my life, work, and personal alike, I know I can turn to him when I need guidance. I dedicate this dissertation to my loving and supporting parents, Karine Kopelman and Howard Love.

ABSTRACT

Courtship in *Drosophila Melanogaster* is an extensively studied and well characterized complex innate behavior with known molecular determinants. We have previously shown that the G-protein Go-alpha and sex-specific signaling in the Blood-Brain Barrier (BBB), influence male innate courtship sustainment towards naïve females. Here, we show that the glial cells that form the BBB modulate male courtship behavior through the dopamine receptor *D2R*. The subperineurial glia cells (SPG) form the BBB by providing a contiguous barrier, connected by septate junctions, at the interface between the hemolymph and the brain. While the neural circuits required to produce scripted actions in the fly, such as the steps for courtship, have been widely investigated, much less is known about how the circuitry and functions underlying the fly behavior is influenced by cell- non-autonomous molecular processes. We have previously shown that male-specific molecules in the BBB regulate male courtship. We identified the *Dopamine-2 like receptor (D2R)* RNA as one of a number of sex-specifically enriched BBB transcripts. D2R knockdown with RNAi or over-expression of D2R with a transgene in the SPG of adult males significantly reduces courtship. Knockdown or overexpression of D2R with ubiquitous neuronal drivers or SPG knockdown specifically during development has no effect on the courtship index. *D2R* mutant flies have courtship defects that can be rescued by expression of wildtype D2R in the BBB of adult males. D2R likely signals through Go and beta-arrestin, to maintain full male courtship levels. Our results indicate an expanded role for dopamine signaling in the glial cells that surround the brain and provide a critical time frame for its action in behavior.

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Chapter 1: Introduction

1.1 Biology of animal behavior

Animals depend on their brains' ability to integrate sensations received from their environment and consolidate internal states to produce behaviors that allow them to survive and reproduce in nature (Wimer, 1985). These behaviors are broadly characterized as innate and acquired. In *Drosophila*, male courtship is an innate social behavior (Robinson et al., 2008). It is genetically programmed and hard-wired in the neural circuitry of the fly brain during neuronal development and initiated by maturation, resulting in the scripted action produced by mature males towards females to entice them to mate (Ewing, 1983; Greenspan et al. 2000; Manoli et al. 2005). The male courtship behavior also has a learned component that is acquired through social experience, allowing the male to modify when he produces the display (Tierney, 1986; Griffith et al., 2009).

The interplay between genes, hormones, and the environment in directing the development of the nervous system and the production of behaviors is complex, multifaceted, and context dependent (Mann et al., 1994; Hiller-Sturmhofel and Bartke, 1998). *Drosophila* serves as an excellent model organism (Sokolowski, 2001; St Johnston, 2002). The flies are genetically tractable and easy to breed, maintain, manipulate, and observe in laboratory settings. The genetic tools at hand to investigate the biological mechanisms of action in *Drosophila* are unmatched, allowing for temporal and spatial control of gene expression and neural circuit action, easily exchangeable genetic cassettes, and the production of mutants and screens. (Robertson et al., 1988; Parks et al., 2004; Groth et al., 2004; Venken et al., 2011; Brand and Perrimon, 1993; Owald et al., 2015; Kim et al., 2017; McGuire et al. 2004). Many behavioral assays are also well established in Drosophila (Homhk et al., 1980).

1.2 Courtship in *Drosophila*

Genes from a range of well-understood processes, including membrane trafficking and circadian rhythms are involved in producing the species-specific courtship display of *Drosophila melanogaster.* Courting behavior is genetically programmed during development by the sex determination system (Greenspan and Ferveur, 2000). Vision is the dominant sensory cue that determines the initiation of courtship pursuit, and chemosensory cues play into the length the male will pursue the target (Krstic and Noll, 2009). Courting males depend on the integration of gustatory and behavioral feedback from the courted animal in the absence of visual cues to determine courtship status (Agrawal et al., 2014). Courtship plasticity is impacted through sensory stimulation by modifying the male's activity in processes of associative memory, habituation, and sensitization with their environment that allows the male to learn from their own performance and target appropriate mates for courtship display and reproduction (Griffith and Ejima, 2009).

The steps of male courtship in *Drosophila* include:

1. Orientation toward the potential mate (and following as the female moves away)
2. Tapping of the abdomen with forelegs (to receive gustatory cues that feed into the courtship drive)
3. Singing (species-specific sine and pulse sound waves generated by the movement of the wing at an angle perpendicular to the body of the male facing outward and oriented toward a position flanking the side the female is being pursued from)
4. Licking of genitalia with his proboscis

5. Attempted copulation or copulation with a receptive female. A receptive female will slow her retreat as a result of the courtship display and allow copulation (Figure 1).

Males produce the display to entice females to mate. The motor action program for courtship is elicited by specific dopaminergic circuit activity requiring successively higher thresholds of stimulation for the activation of a pair of neurons that direct the neuronal circuit that controls the sequence of independent actions that lead into each other to produce the ritualized goal-directed behavior that is successive but persists cumulatively as a drive in mature males (Mckellar et al., 2019; Cobb, 2019).

Male mating drive in *Drosophila* decreases transiently and cumulatively as reproductive capacity is depleted by successive copulations, and the drive weakens with age. These behavioral properties coincide with dopamine signaling dynamics in the neural circuit that relays sensory information from the female to the male to stimulate courtship (Kuo et al., 2015). Dopamine levels in the neural circuit are depleted as a function of copulations. Copulation-reporting neurons detect matings and induce satiety. A recurrent excitation loop promotes recovery from satiety over days by elevating a dopamine signal through CREB that transcribes the inhibitory channel subunit TASK7. Courtship drive can be restored in aged males by increasing dopamine production in the dopamine neurons that are involved in initiating and sustaining courtship (Zhang et al., 2016; Zhang and Rogulja, 2019).

In addition to the dopamine circuit, the circadian system is important for male courtship drive in *Drosophila*. Circadian oscillators in the brain and antenna of males are required for a distinct nocturnal intrinsic locomotor output pattern found in socially interacting male and female flies. *Sex-specific enzyme 1* (*sxe1*) is a sex-specifically expressed transcriptional

regulator and a circadian gene that is required for normal mating success in males throughout the diurnal cycle (Fuji et al., 2007; Fuji et al., 2008).

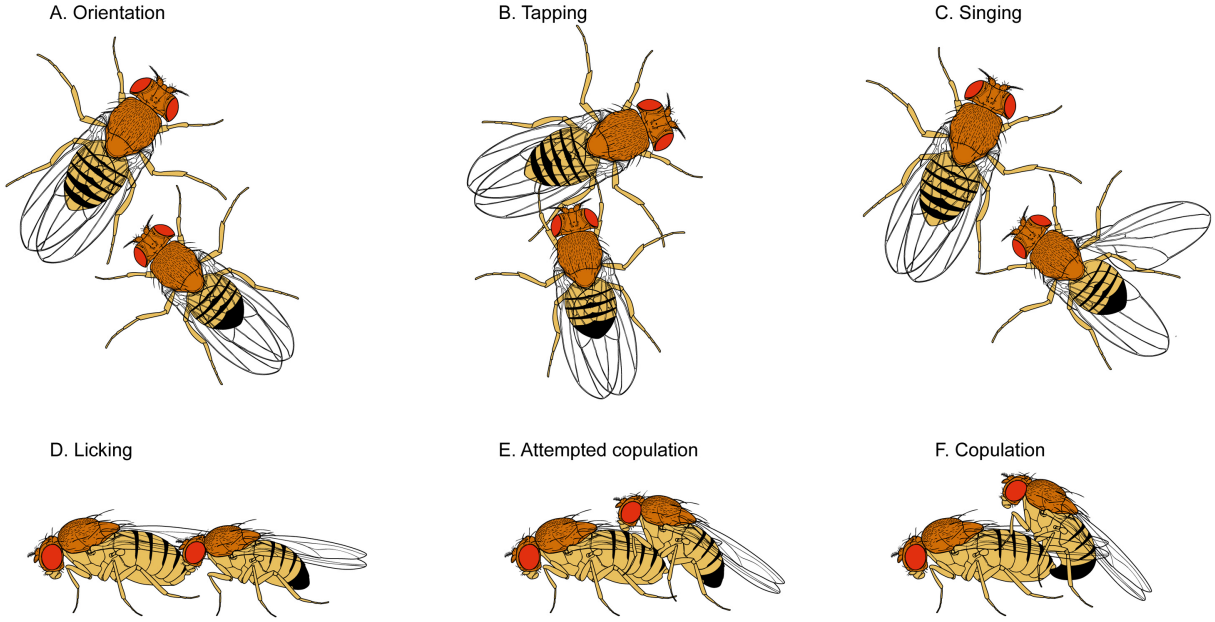

Figure 1: Courtship in *Drosophila*

Healthy mature males produce the courtship steps in order and persistently towards females until mating is achieved or the drive is lost.

1.3 Molecular determinants of sex-specific behavior

1.3.1 Genetic control of sex determination in *Drosophila*

In mammals, steroid sex hormones likely enable the display of sexually dimorphic behaviors by patterning the central nervous systems through the activation of master

regulators of sex determination that initiate sexual differentiation through cascades of sexually dimorphic gene expression. Epigenetic and transcriptional mechanisms that control the development of sex-specific behaviors may be evolutionarily conserved in the neuronal circuits of mammals, flies and worms (Knoedler and Shah, 2018). *Drosophila* male mating behavior is a complex instinctive response that is hardwired in the fly brain through the somatic sex determination pathway (Arbeitman et al., 2004). The control of innate courtship display in the male flies has been mapped to distinct clusters of neurons. These sets of neurons express sex-specific variants of the zinc finger transcription factor encoded by *fruitless* (Figure 2), a gene called *doublesex* is also involved (Dauwalder, 2011).

fruitless (*fru*) and *doublesex* (*dsx*) are spliced in a male-specific pattern in males and go on to pattern the sexually dimorphic circuitry in males likely by regulating chromatin states that coordinate gene transcription programs (Heinrichs et al., 1998; Goodwin et al., 2000; Rideout et al., 2010; Sebastian et al., 2010). One example of this includes midline crossing by gustatory receptor neurons in males that is initiated by *fru* and *dsx*, likely mediated by the activation of *roundabout* (*robo*) in these neurons (Mellert et al., 2010).

Masculinization of FruM-expressing neurons in females is sufficient to elicit courtship behaviors in females and is necessary in males to produce the behavior (Yamamoto et al., 2013; Kimura et al., 2005; Manoli et al., 2005). The *fru* positive P1 interneuron cluster likely forms the core portion of the male courtship circuitry. Activation of P1 neurons by optogenetic or thermogenetic means and motion cues is jointly sufficient to stimulate courtship in males (Pavlou and Goodwin, 2013; Yu et al., 2010; Pan et al., 2012).
The antagonistic chromatin regulators histone deacetylase 1 and heterochromatin protein 1a masculinize or demasculinize single neurons in concert with FruM (Sato and Yamamoto et

al., 2014). *Hector* is a G-protein coupled receptor required for courtship that is expressed in a subset of *fru*-positive antennal glomeruli (Li et al., 2011). During metamorphosis, downstream effectors of FruM remodel the nervous system for adult function likely through the ecdysone receptor that is activated by steroid hormone 20-hydroxyecdysone (ecdysone) in P1 neurons and affect the size of two antennal lobe glomeruli (Dalton et al., 2009). These findings indicate a potential signaling role for G-protein coupled receptors and nuclear hormone receptors in fruitless neurons and their effectors that is required for normal courtship.

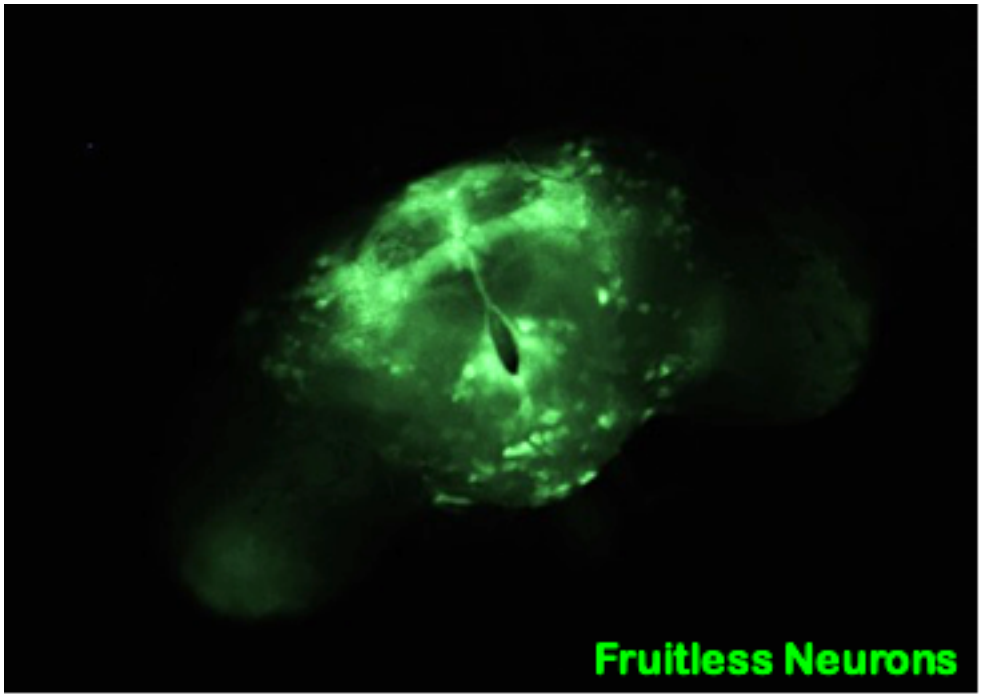

Figure 2: Fruitless neurons in the fly brain

Fruitless neurons express the male-specific version of Fru and modulate the development and display of male courtship behavior. A dissected fly brain expressing a GFP reporter in *fruitless* neurons is shown.

1.3.2 Influence of circulating factors on *Drosophila* courtship

The timing of metamorphosis is initiated and terminated by changing levels of circulating insect hormones including ecdysone and juvenile hormone (Sanburg et al., 1975; Williams, 1956). Ecdysone is produced by the prothoracic gland and juvenile hormone is a gonadotropic hormone produced by the *corpora allata* (Richard et al., 1989; Whitmore and Gilbert, 1972; Bownes and Rembold, 1987). These insect hormones are protected by carrier proteins as they circulate in the hemolymph to reach hormone receptor targets in the brain to regulate development and reproductive maturation (Ishimoto and Kitamoto, 2010). Signaling changes and hormone dynamics that direct sexual maturation has long-lasting effects in mature adults. JH has a sexually dimorphic role in modulating adult behaviors in *Drosophila* through a sex specific interaction with dopamine by an indirect mechanism during maturation (Argue et al., 2013). Gonadotropic hormones and dopamine also interact to affect brain development and adult functions in mammals, suggesting an evolutionarily conserved mechanism for the programming of adult behaviors during maturation.

Takeout is a male enriched carrier protein under circadian clock transcriptional regulation that is produced and secreted by the fat body tissue in the head. The fat body functions as a nutrient sensor through TOR signaling to regulate nutrient dependent growth and development. The fat body retains endocrine and storage functions analogous to the vertebrate liver (Benito et al., 2010; So et al., 2000; Colombani et al., 2003).

The sex-determination system regulates *takeout* through *fru* and *dsx* and its production and secretion by the fat body is required for normal levels of courtship in mature adult males. Takeout is a putative JH-binding protein regulated by the sex determination system, the

circadian clock and nutrient responsive hormones that influence lifespan and male courtship behavior (Dauwalder et al., 2002; Galikova and Flatt, 2010; Saurabh et al., 2018).

JH also influences lifespan and promotes male mating behavior through its action on the nuclear hormone receptor Methoprene-tolerant (Met), which is likely involved with protein synthesis in the male accessory glands and the post-eclosure development of the adult fat body (Yamamoto et al., 2013; Wilson et al., 2003).

Ecdysteroids modulate female sexual precopulatory behavior. Reducing ecdysone receptors in *fruitless* neurons reduces female rejection behaviors and ecdysone-less females perform male-like courtship behaviors. Ecdysteroids also modulate the male courtship behavior and ecdysone deficiency in males induces elevated male-male courtship behavior. Ecdysteroid action likely increases the probability that males will display courtship behaviors (Ganter et al., 2012; Ganter et al., 2012).

Nutrient dependent phenotypic plasticity controls the production of exaggerated male specific traits in insects through DSX by increasing sensitivity to JH in males and decreasing sensitivity in females. This can be seen in specific sexual traits such as the mandible growth of male stag beetles that are used as ornaments and weapons of sexual selection. This sensitivity to JH is perhaps mediated by *dsx* control of Takeout and related carrier proteins that export, protect, and transport JH and other insect hormones like ecdysone, while JH synthesis is being mediated by nutrient status (Hiroki et al. 2014).

Male-produced sex pheromones induce increased attraction to food and sex in female flies through the upregulation of insulin-signaling in the DA1 glomerulus by activating the olfactory centre in the insect brain that includes the antennal lobe. This allows the female to

physiologically respond to sex pheromones according to nutritional state and sexual receptivity (Lebtreton et al., 2015).

In mosquitoes, starvation decreases JH synthesis via a decrease in insulin signaling in the *corpora allata* (Perez-Hedo et al., 2014). Takeout expression is induced by starvation and this process is blocked in *Drosophila* central clock mutants. This circadian output pathway conveys temporal and food status information to sex and feeding-relevant metabolisms and activities in the flies (Sarov-Blat et al., 2000). Takeout mutants lack sexual dimorphism of locomotor activity and have a defect in food intake regulation as a result of desensitization of takeout gustatory neurons to sugar following starvation (Meunier et al., 2007). Takeout may act by modulating the circulating JH level. JH plays a role in regulating adult male courtship (Wijesekera et al., 2016). Sex determination in the fat body also feeds into regulating courtship through the production and secretion of takeout (Lazareva et al., 2007).

Mating itself alters gene expression patterns in *Drosophila* male and female heads by changing the expression of fat body-enriched genes that are downstream targets of the sex-determination hierarchy including *female-specific independent of transformer* (*fit*) and Juvenile hormone esterase (*Jhe*), which are necessary for robust male courtship behavior (Ellis and Carney, 2010).

The ability of hormones and nutrients or sex responsive factors to affect brain development and activity requires them to interact and communicate with the Blood-Brain Barrier (BBB). For example, the reactivation of neural stem cells following first instar molting in *Drosophila* requires the production and secretion of a nutrient responsive factor in the fat body that goes on to stimulate the production and release of insulin in the BBB into the brain to activate insulin receptors on quiescent neuroblasts, reactivating their growth and divisions during

larval development (Garcia-Caceres et al., 2016). In mammals, insulin signaling in astrocytes regulates CNS glucose sensing and systemic glucose metabolism via regulation of glucose uptake across the BBB, again this suggests an evolutionarily conserved mechanism for communication between the brain and circulating factors to regulate brain development and the production of social behaviors (Sousa-Nunes et al., 2011).

1.3.3 Physiology of the *Drosophila* Blood-Brain barrier

The intricate interplay between neuronal and glial cell types produces the functions of a complex nervous system. All organisms with a complex nervous system have a blood-brain barrier (BBB) that provides efficient insulation for the brain. The BBB provides protection for the brain from entry of solutes, metabolites or pathogens; this boundary maintains extracellular ion homeostasis in the CNS and supplies it with nutrients and metabolites for the efficacy of neuronal function, (Stork et al., 2008). The diffusion barrier forms extensive tight junctions between endothelial cells in higher vertebrates and between surface glial cells in lower vertebrates and invertebrates. In *Drosophila,* at least seven morphologically distinct glial cell classes can be distinguished and two of these glial cell types form the BBB. The outer layer of the BBB is formed by the perineurial glia (PG) that keep out large protein sized molecules. The subperineurial glia (SPG) form the inner tight barrier that prevents paracellular diffusion of small molecules and ions by forming and maintaining septate junctions between the cells (Figure 3). The septate junction components are largely conserved between vertebrates and *Drosophila*, including mechanisms of paracellular barrier maintenance through G-protein coupled receptor signaling and orchestration of synchronized neuroblast proliferation by BBB gap junctions that coordinate functions through all layers of

the compound barrier structure, analogous to signaling between the layers of the vertebrate neurovascular unit (Hindle and Bainton, 2014; Limmer et al., 2014).

The SPG cells form the blood-brain barrier during embryogenesis and terminally differentiate once the layer is established. The SPG cells are polyploid, allowing them to continue expanding and endo-replicating as the brain grows, thus coordinating the action of the BBB with the brain (Unhavaithaya and Orr-Weaver, 2012; Orr-Weaver, 2015). This type of coordinated polyploidization is likely a conserved strategy for tissue growth during organogenesis. A variety of animals and plants utilize polyploidy functions to control organ size, the size and function of specific tissues within an organ, differentiated properties of cells, wound healing, tissue regeneration, and other physiological functions (Fox and Duronio, 2013).

The surface glia contain many ATP-binding cassette (ABC) and solute carrier (SLC) transporters, cell adhesion molecules, metabolic enzymes, signaling molecules, and components of xenobiotic metabolism pathways that are evolutionarily conserved between flies and mammals (DeSalvo et al., 2014; Hatan et al., 2011). GPCR-dependent dynamic actin structures in addition to signaling through the *moody* GPCR, is required for blood-brain barrier formation, coinciding with elevated calcium levels in the BBB that produce synchronized waves that move between the cells through gap junctions (Schwabe et al., 2005). Sex determination in the BBB influences courtship. Sex-specific factors and Galpha(o) signaling from GPCR's in the BBB is required for male courtship behavior (Hoxha et al., 2013). Besides its involvement in courtship regulation, *moody* is also involved in regulating behaviors in response to cocaine and alcohol, and in regulating BBB permeability. *Mdr65* in the BBB has an orthologous function to the vertebrate ABC transporters that produce the

xenobiotic exclusion properties found in the vertebrate vascular endothelium, highlighting the evolutionarily conserved physiological functions of the BBB (Bainton et al., 2005; Mayer et al., 2009).

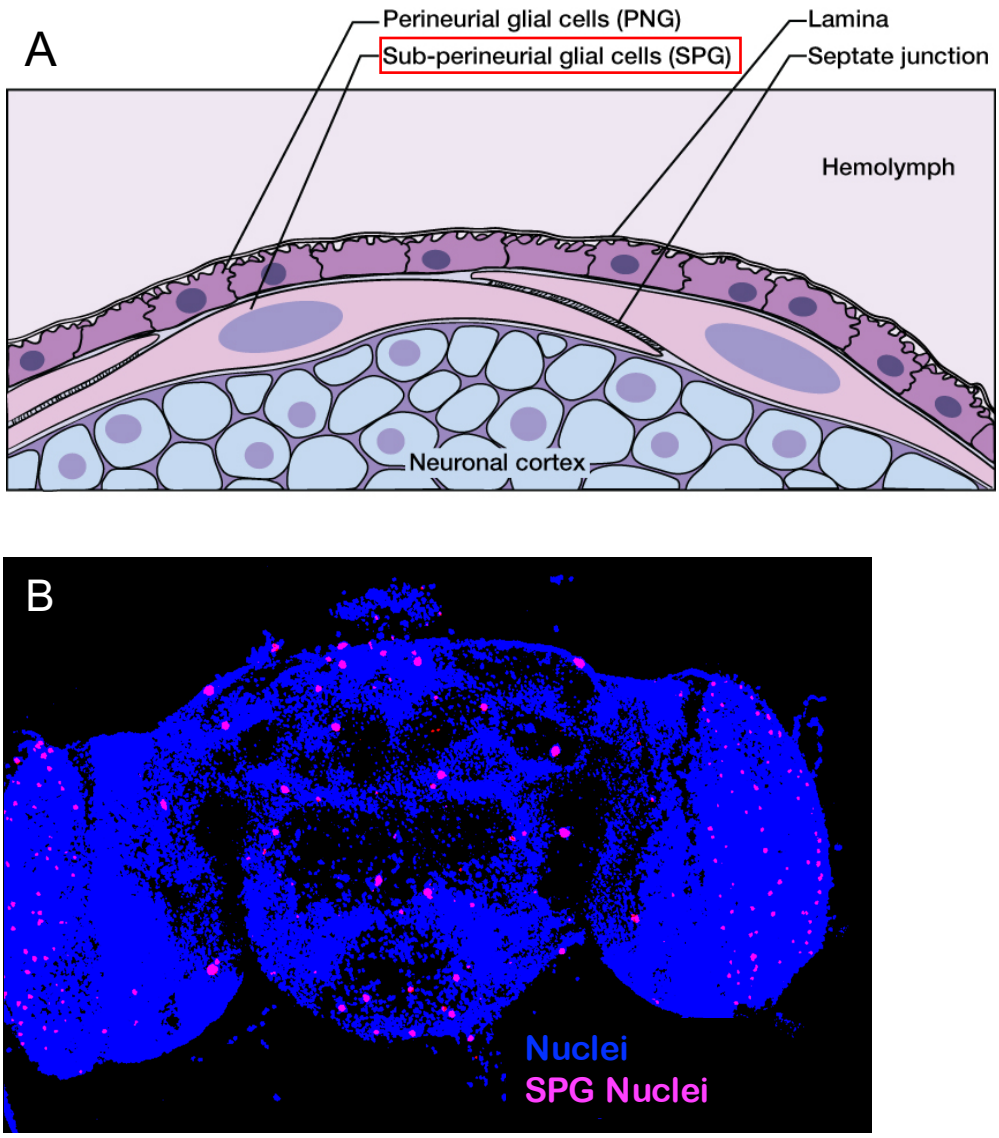

Figure 3. BBB schematic and Z stack projection of BBB nuclei surrounding the *Drosophila* brain. (A) The BBB consists of two layers of surface glial cells including the PG and the basal SPG that forms septate junctions between the cells to create the barrier function that protect the neuronal cortex, schematic is from Mike De La Flor. (B) Isolated fly brain with SPG nuclei marked by a reporter (pink). The nuclei of brain neurons are in blue. SPG consist of only a few hundred cells that surround the brain. They are large, flat, multinucleated and polyploidy in nature but terminally differentiate in establishing the BBB.

1.4 Biology of the dopaminergic system

1.4.1 The Dopaminergic system in *Drosophila*

From learning and sleep to courtship and aggression, dopamine modulates a variety of animal behaviors. In mammals and flies, dopamine plays a role in modulating specific behavioral responses to cocaine, nicotine, or ethanol (Bainton et al., 2000). Methamphetamines suppress sleep and promote active wakefulness by inducing influx of dopamine through the dopamine transporter (Kahlig et al., 2005). Complex behaviors degenerate when dopamine levels are too high or low and simpler behaviors - sleep and locomotion, for example - show a graded response to changes in dopamine levels (Andretic et al., 2005). Male-male courtship is enhanced by increasing dopamine levels, likely by altering the sensory perception of other males (Liu et al., 2008). Neuromodulation involving dopamine also plays a role in internal state-dependent odor processing and perception in the fly olfactory system, as well as in learning and memory (Sayin et al., 2018; Aso, 2016; Qi, 2014).

The dopamine ontogeny hypothesis for schizophrenia proposes that transient dysregulation of the dopaminergic system during brain development increases the likelihood of this disorder in adulthood. As a model for this hypothesis, increasing or decreasing dopamine neuron activity during fly brain development permanently impairs behavioral responsiveness in adults (Ferguson et al., 2017; Henn, 1978). The modulatory role for dopamine in the production of schizophrenic symptoms may be indirect, as fine structures of dopaminergic fibers indicate that most presynaptic boutons are not in direct contact with postsynaptic neuronal membranes, suggesting a role for auto receptor activity in glial cells. In support of an indirect glial effect,

the schizophrenia susceptibility gene *dysbindin* regulates the dopamine metabolic enzyme Ebony in glial cells to maintain normal locomotion and mating orientation in the flies (Shao et al., 2011).

The dopaminergic modulation of sucrose acceptance behavior is linked to the control of sugar sensing by dopamine signaling in *Drosophila* (Marella et al., 2012; Inagaki et al., 2012). Dopamine has a conserved role in regulating goal directing behaviors, motivation control and setting arousal thresholds in the brain (Grace et al., 2007; Bromberg-Martin et al., 2010; Kume et al., 2005). *Drosophila* is an important model system to gain a better understanding about how neuromodulators regulate motor tasks and cognition in humans (Van Swinderen and Andretic, 2011; Friggi-Grelin et al., 2003; Yamamoto et al., 2014).

1.4.2 Molecular mechanism of dopamine receptor function

Dopamine receptors belong to the superfamily of G protein-coupled receptors (GPCRs) that produce the majority of their effects by binding to heterotrimeric G proteins which consist of an alpha subunit, and a dimeric beta and gamma subunit. There are 5 subtypes of the Dopamine receptors in people, $D_1 - D_5$. The receptors fall into two families: the D_1 and D_5 receptors stimulate adenylyl cyclase to produce cAMP. Dopamine D_2 D_3 and D_4 receptors produce the opposite effect and inhibit the activity of adenylyl cyclase and the amplification of PKA phosphorylation activity. This occurs through the activity of their respective $G\alpha_s$ and $G_{\alpha i/o}$ following the binding of dopamine, or in other cases as a result of constitutive receptor activity, perhaps as a consequence of GPCR heteromer networks (Beaulieu et al., 2015). These processes go on to activate specific signaling pathways (Kienast and Heinz, 2006; Le

Foll et al., 2009). Dopamine receptors also activate cAMP independent pathways including the recruitment of beta arrestin that acts to desensitize the receptors and also initiates its own signaling pathway leading to activation of extracellular signal-regulated kinases (Beaulieu and Gainetdinov, 2011). Dopamine receptors are implicated in numerous neurological processes and modulation of neuroendocrine signaling. Dysfunction of dopaminergic neurotransmission in the CNS is implicated in a variety of neuropsychiatric disorders, including Parkinson's disease, ADHD, schizophrenia, and drug and alcohol dependence. Synthetic agonists and antagonists for dopamine receptors are often used to treat these disorders (Mustard et al., 2005).

Thermogenetic stimulation of *fruitless* neurons elicits courtship from males even without a female to target (Kohatsu et al., 2011). The *fruitless* circuit that elicits courtship produces a transient rise of Ca^{2+} in dopamine receptor 2 (DR2) positive P1 neurons, as the male is stimulated to perform courtship by touching his foreleg tarsus to the female's abdomen (Kimura et al., 2008). The mushroom body D_1-dopamine receptor (D_1R) is implicated in mediating courtship learning in the flies (Zhang et al., 2016). The dorsal fan-shaped body regulates sleep via a switch between active and quiescent states in sleep promoting neurons that mediate its action through dopamine stimulation of *dopamine receptor 2* (*DR2*) receptors that control potassium conductance in these cells (Lim et al., 2018; Pimentel et al., 2016). The *Drosophila* dopamine/ecdysteroid receptor *DopEcR* shows homology to the vertebrate "gamma-adrenergic receptors" and may be responsible for some of the rapid, nongenomic actions of ecdysteroids, during both development and signaling in the mature adult nervous system (Srivastava et al., 2005; Guadarrama-Bazante and Rodriguez-Manzo, 2019).

1.4.3 The *Drosophila* Dopamine 2-like Receptor

In general, there are two major forms of Dopamine D2 type receptors, long and short, that are produced as a result of alternative splicing. The long isoform is present on the postsynaptic side, receiving input from dopaminergic neurons to stimulate the signaling pathways initiated by dopamine (De Mei et al., 2009). The short isoform is present on dopaminergic cell bodies and axons, likely acting as an autoreceptor by coupling with the dopamine transporter to regulate levels of dopamine in its environment (Khan et al., 1998; Usiello et al., 2000; Hearn et al., 2002). The *Drosophila dopamine 2-like receptor (D2R)* has eight unique isoforms, seven of these fall into the long form category with differences in the third intracellular loop, while one is the short variant that is missing the third intracellular loop portion all together as is found in mammalian D2R (Hearn et al., 2002). Expression studies in mammalian HEK392 cells suggest dopamine as the dominant ligand for D2R (Hearn et al., 2002). Partial knockdown of *D2R* reduces general locomotion in the flies that can be rescued by administering the D2R agonist bromocriptine (Draper et al., 2007; Wiemerslage et al., 2013). Hypomorphic and null *D2R* mutants have deficits in long term memory (Scholz-Kornehl and Schwärzl, 2016).

1.5 Research Questions and Approach

This project examines the role of D2R in the BBB, specifically addressing its role in male courtship behavior. Using genetic tools and the Gal4/UAS/Gal80ts system I specifically knocked down or overexpressed D2R in the BBB and studied the effect on courtship. This approach allowed me to focus on the adult physiological role of D2R. I characterized the courtship defects of available D2R mutants and tested whether they could be rescued by expression of functional D2R in the BBB. To examine which signaling pathways mediate D2R action for mating behavior, I performed genetic interaction studies, specifically testing the role of beta-arrestin. I further laid the groundwork for pharmacological studies of D2R action and downstream pathways.

Chapter 2: *Drosophila* as a Model to Study the Blood-Brain Barrier

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Love C.R., Dauwalder B. (2019) *Drosophila* as a Model to Study the Blood-Brain Barrier. In: Barichello T. (eds) Blood-Brain Barrier. Neuromethods, vol 142. Humana Press, New York, NY.

1. Summary

The *Drosophila* blood brain barrier (BBB) has been shown to be largely analogous in structure and function to the vertebrate BBB. Thanks to the genetic tools available for this organism, *Drosophila* is uniquely suited to study BBB physiology and function, with high relevance for mammalian function. In this chapter, we discuss targeting strategies to specifically mark and manipulate BBB cells, how to test BBB barrier integrity, and methods to isolate single BBB cells.

2.1 Introduction

Like in vertebrates, the insect blood brain barrier protects the brain from components of the circulating fluid and allows selective uptake of nutrients and other important molecules. A big difference to vertebrates lies in the fact that insects do not have blood vessels but an open circulatory system. The hemolymph, the circulating fluid, is moved through the body of the animal by the pumping action of the heart and bathes all organs – except for the nervous system that is protected by the BBB. The insect hemolymph contains particularly high K+ concentrations that would be detrimental to neuronal function. Due to the absence of blood vessels, the blood brain barrier surrounds the entire brain like a tight "cap" (Figure 4). The insect BBB is best studied in *Drosophila* where it has been shown to be analogous in structure and function to the vertebrate BBB. In contrast to vertebrates, the insect BBB is formed by two layers of glial cells, an outer layer called Perineurial glial (PG) cells and the inner layer, the subperineurial glial cells (SPG) with the septate junctions that form the tight barrier. The SPG barrier forms early in development and its cells do not divide in later stages. Therefore, the number of SPG cells is low. To adapt to the growing brain, the cells flatten and become polyploid (Unhavaithaya and Orr-Weaver, 2012). Despite differences between the insect and

the mammalian BBB, it has been shown that components that form the barrier (for example neurexins and forms of claudins), as well as many of the functional properties are shared between species. Several excellent reviews and recent papers discuss these aspects in detail (DeSalvo et al., 2014; DeSalvo et al., 2011; Hindle and Bainton, 2014; Limmer et al., 2014; Mayer et al., 2009; Stork et al., 2008). Given the unparalleled genetic tools available in Drosophila, this tractable organism is uniquely suited to study BBB physiology and function, with high relevance for mammalian function (Hindle et al., 2017). These studies go beyond mere barrier function, with novel insights starting to emerge about physiological processes inside BBB cells that influence neuronal development, adult neuronal function and sex-specific behavior (Bainton et al., 2005; Hoxha et al., 2013; Chell and Brand, 2010; Speder and Brand, 2014).

In this article we will describe several methods that are being used to examine and manipulate Drosophila BBB function. We will discuss the specific labeling of BBB cells using the Gal4/UAS/Gal80ts system, the dissociation and selection of BBB cells, and a method to examine BBB integrity.

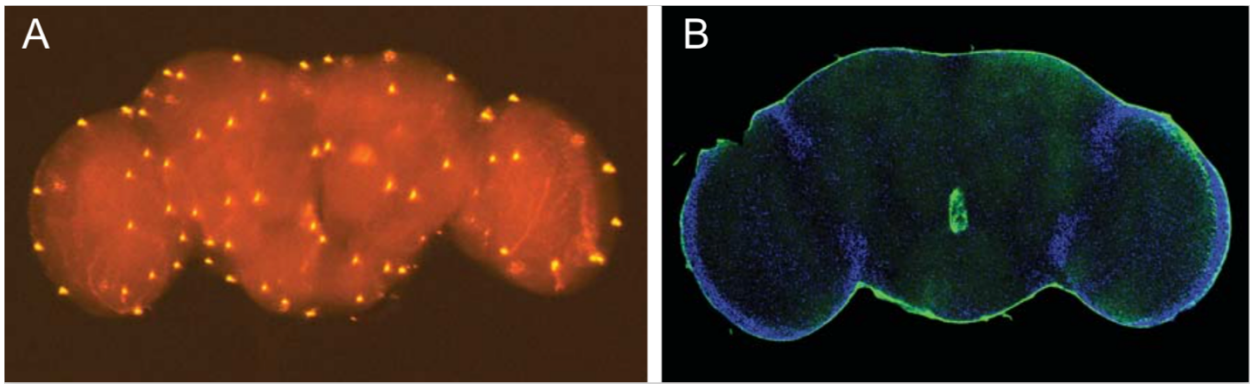


Figure 4: Isolated adult *Drosophila* brains with BBB visualized

Dorsal is on top. (A) Nuclei of SPG cells are labeled by expression of dsred. Genotype: Mdr-Gal4/+; UAS-dsRed/+. SPG cells are large and flat, and their number is low. (B) Both layers of the BBB (PG and SPG cells) express a genomically encoded indy-GFP fusion protein, visualized by immunohistochemistry (green). DNA is shown in blue (the majority of the nuclei seen are neuronal). An optical confocal section is shown to illustrate the tight barrier layer that surrounds the entire brain. The opening inside the brain with a BBB layer is the oesophageal foramen.

2.2 BBB labeling and manipulation using the Gal4/UAS System

One of the most powerful tools in Drosophila research, the binary Gal4/UAS system allows the manipulation of cells in a tissue-specific and temporally restricted manner (Figure 5A) (Brand and Perrimon, 1993; McGuire et al., 2004). It allows the expression of desired molecules in the cells of choice and is efficiently used to label cells with fluorescent molecules, or to manipulate cells by expression of interfering/silencing RNAs (RNAi) or any transgene of interest. The Gal4/UAS system makes use of the yeast transcription factor Gal4

that binds to the UAS sequence (upstream-activating-sequence) and activates transcription of sequences downstream of UAS. Gal4 and the UAS constructs are introduced into the flies as independent transgenes, and strains containing either element can be crossed as desired. The system allows expression of sequences of choice (such as fluorescent proteins, or interfering RNAs) in any cells of choice, as long as a specific promoter sequence is known that directs expression to the targeted cells. Temporal control of expression is achieved by the simultaneous presence of a transgene that ubiquitously expresses a temperature-sensitive inhibitor of Gal4, Gal80ts (McGuire et al., 2004) (Figure 5B). When the animals are kept at 18 ^{o}C, Gal80ts represses Gal4 and no expression occurs from the UAS target. Upon shifting of the flies to temperatures between 29 – 32 ^{o}C, Gal80ts is inactivated and Gal4 can begin transcription of the sequences downstream of UAS. The use of a conditional expression system to manipulate transcript levels at defined times allows the study of the temporal requirement of a gene. This is also valuable for genes that might have functions in both development and adulthood.

2.2.1 Materials

A critical feature for specificity of expression is the promoter used to drive expression of Gal4. Several BBB-Gal4 transgenic lines have been generated and described. Among them, the moody-Gal4 (also called SNG-Gal4 or SPG-Gal4) lines generated by Bainton et al., 2005 have been widely used. Several sublines exist that contain the SNG-Gal4 insertion at different chromosomal location and that may vary in the degree to which they express in a few other cells outside of the SPG. As a standard procedure Gal4 lines should be crossed to a UAS-fluorescent protein to examine the expression pattern of a particular Gal4 line. Our lab has

recently generated a Mdr65-Gal4 line using the promoter of the SPG-specifically expressed Mdr65 gene, a P-glycoprotein homolog (Figure 4A; unpublished). In comparison, a genomic fusion construct that leads to the expression of the indy protein fused to GFP (indy-GFP) is expressed in both SPG and PG cells (as shown in Figure 4B). Line 9-137-GAL4 (Ulrike Heberlein, Janelia Farm Research Campus, VA) is expressed in both layers of the BBB (DeSalvo et al., 2014). A different transgenic line carrying indy-Gal4 has recently been described that is specifically expressed in PG cells (Parkhurst et al., 2018). These fly lines can be obtained from the labs that created them.

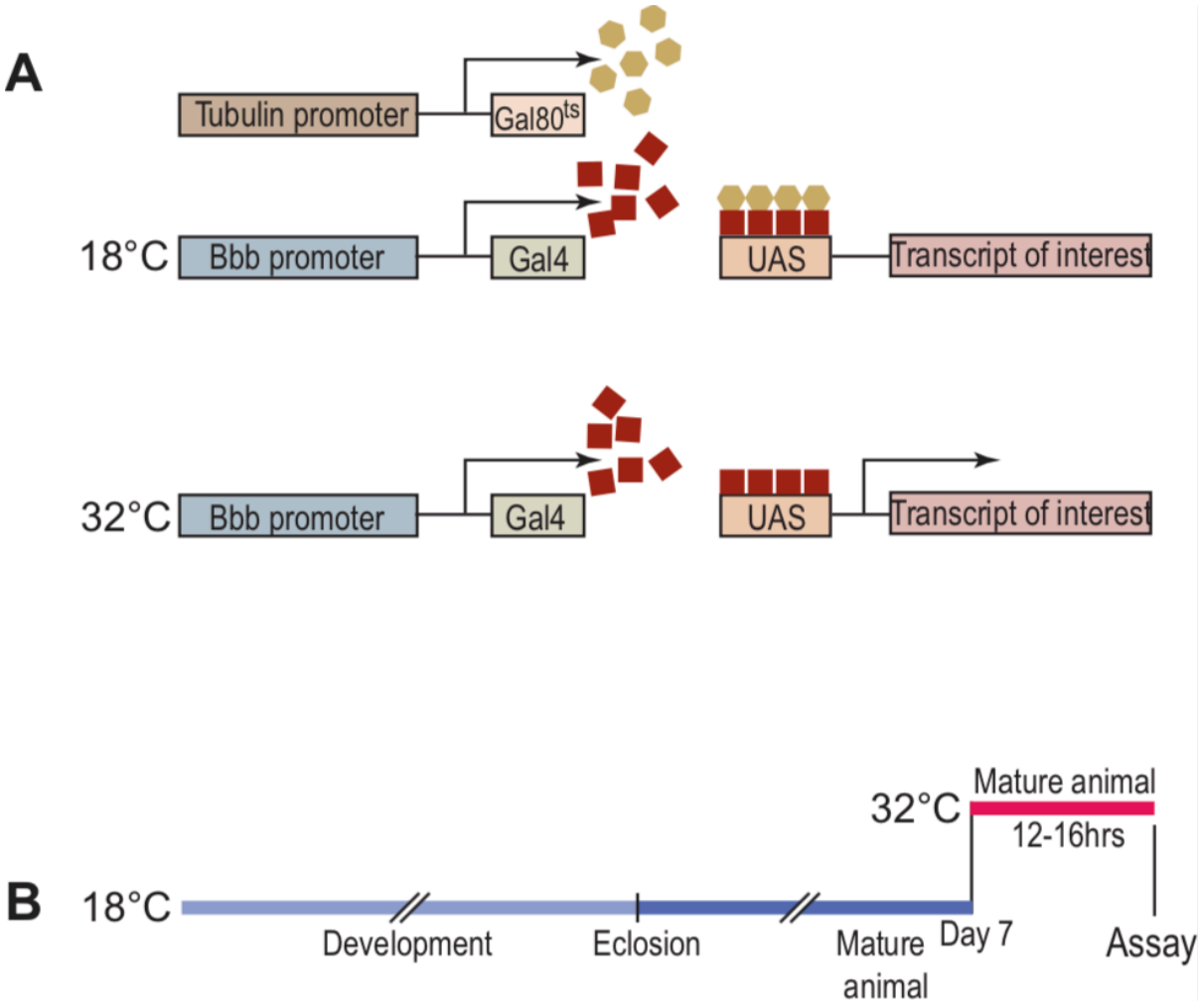


Figure 5: The Gal4/UAS/Gal80ts system allows temporally controlled expression of sequences of choice in the cells of choice. (A) Gal4 expression is directed to the BBB by a BBB-specific promoter. There, Gal4 binds to UAS and leads to the transcription of the downstream sequence. However, at the same time, a ubiquitous tubulin promoter guides direction of the temperature-sensitive Gal80^{ts}, an inhibitor of Gal4. At 18 ^{o}C, Gal80 ^{ts} is active and inhibits Gal4 activity and transcription is blocked. To release this block, animals are shifted to 32 ^{o}C. At this temperature Gal80 ^{ts} becomes inactive, and Gal4 can initiate transcription form the UAS promoter. (B) Protocol to manipulate BBB cells specifically in mature adult flies using the approach described in (A).

2.3 Assessment of barrier integrity

Like in mammalian systems, the integrity of the BBB is tested by injection of small-molecular-weight molecules into the circulatory system and assessment of their exclusion from the brain. 10 kD Dextran coupled to fluorescent Texas Red (Dextran-TR) is effectively excluded from the brain in flies with an intact BBB. Since flies have an open circulatory system, the dye can be injected into the fly's abdomen. Following injection, the dye circulates throughout the body of the animal and will accumulate at the BBB, excluded from the brain, where its accumulation can be visualized following brain dissection. This system has also successfully been used to screen for new mutants that affect BBB integrity, be it in development or in adults. When the BBB is leaky, the dye will not be excluded and enter the brain and fluorescence can be seen accumulating in the eyes of intact flies when flies with un-pigmented eyes (white eyes) are used (Mayer et al., 2009). Protocols for this assay have been developed by Schwabe et al., 2005 and Bainton et al. 2005

Figure 6A shows accumulation of 10 kD Dextran-TR at the BBB surrounding the brain in wildtype flies. In contrast, flies with a leaky BBB (such as moody mutant flies) have a defective barrier and the dye diffuses through the BBB as shown in Figure 6B.

2.3.1 Materials

- 2.5 mM 10 kDa Texas-Red conjugated Dextran, fixable (Invitrogen D-1863) in H2O.
- 4% Paraformaldehyde (EM grade, Polysciences Inc. #00380-250)
- Microinjector
- Razor blades
- Microscope slides and cover slips

- Double-sided tape
- Vectashield Antifade Mounting Media with DAPI (Vector Laboratories # H-1200)

Brain dissections: Several youtube videos are available with good instructions on how to dissect brains from fly heads. We like https://www.youtube.com/watch?v=j4rVa7JCzdg (Wu and Luo, 2006). It is important to clean the brains up as thoroughly as possible (i.e., remove fat body and trachea). The secret to intact and cleanly dissected brains is a LOT of practice!

2.3.2 Approach

1. Anesthetize adult flies on ice
2. Micro-inject a small amount (20-50 nl) of 2.5 mM 10 kDa Texas-Red conjugated Dextran in H^2O in between sternites or under the scutellum
3. After injection allow flies to recover in regular food vials overnight
4. Anesthetize flies on ice
5. Remove fly heads with a razor blade and drop them into 4% Paraformaldehyde in PBS. Fix heads for 30 mins at room temperature (RT).
6. Remove the proboscis (mouth part) for enhanced penetration of the fixative and incubate for an additional 5 mins at RT.
7. Dissect out the brain. Wash in 1X PBS 3 times for 30 mins each.
8. Mount the brains on a slide with Vectashield mounting media containing DAPI to stain DNA. After a few hrs, seal the coverslip with nail polish.
9. View under a confocal microscope. DAPI- stained cell nuclei are visualized at 405 nm, Texas Red Dextran at 633 nm.

2.3.3 Notes

To mount fly brains on a slide without them being ‘smashed’ by the coverslip, put a small square of double-sided tape onto the slide. With a razor blade cut a small window into the tape into which the brains will be put like in a basket. Add mounting media to the “window”, add the brains, and cover with a coverslip. A little ‘canal’ can be cut from the square so that extra mounting media can drain. The tape will allow the brains some room while the height of the slide plus coverslip is still compatible with imaging under an upright microscope.

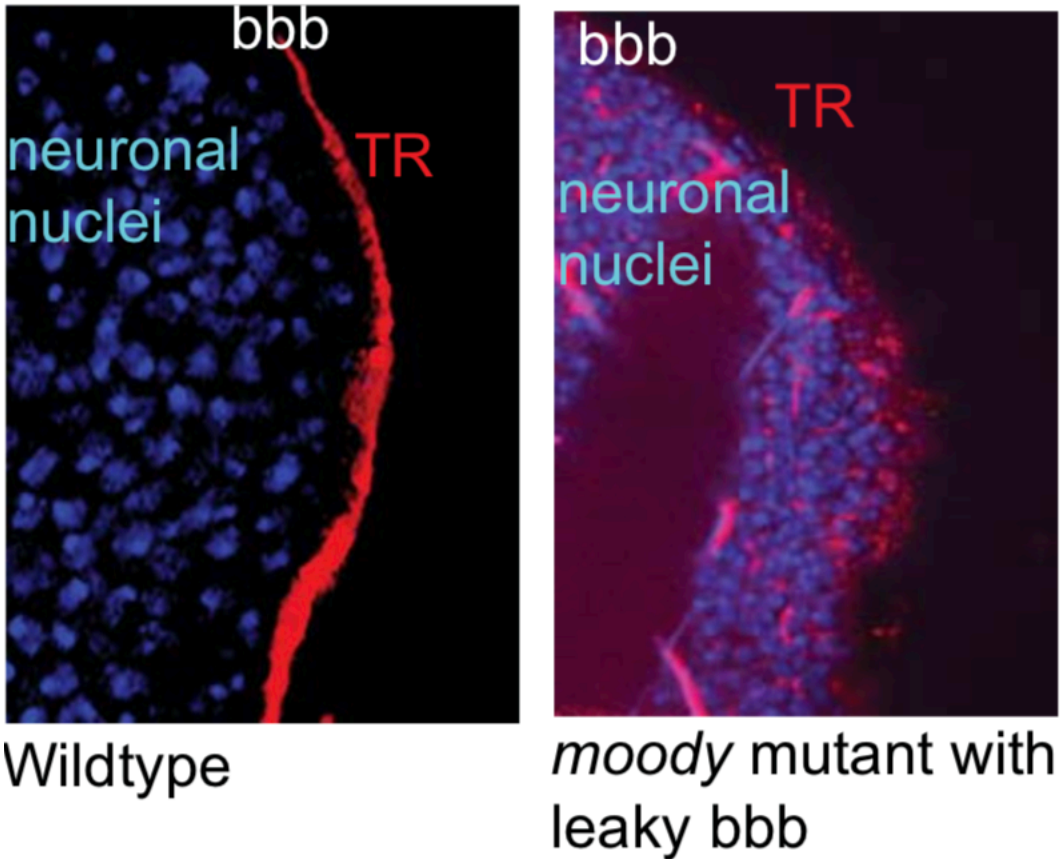


Figure 6: An intact BBB is not permeable to 10kD-TR

(A) Following injection of 10 kD Dextran marked with the Texas-Red (TR) fluorophore, Dextran-TR circulates in the hemolymph and accumulates at SPG cells. The optical confocal section shows accumulation of Dextran-TR (red) at the barrier. Neuronal cell bodies inside the brain are marked with DAPI. (B) moody mutant flies have a leaky BBB (Bainton et al., 2005) and Dextran-TR can be seen entering the brain (Picture reproduced with permission from Hoxha et al. PLoSGenetics, 2013.)

2.4 Isolation of BBB cells

SPG cells can be isolated either manually or by FACS sorting. Both approaches require that the BBB cells are labeled by fluorescence (by using the Gal4/UAS expression

system, for example, as described above) and that brains are dissected prior to isolation of the cells. It is important that surrounding tissues are removed carefully. Below we will describe the sorting of SPG cells following a protocol that was developed by DeSalvo et al. 2014, the most efficient protocol developed to date. Figure 7 illustrates the progression from SPG cells on the brain to isolated cells. It is worth noting that since SPG cells are large and very flat, once removed from their neighbors, their shape changes.

2.4.1 Isolation of fluorescently marked BBB cells by FACS sorting

Cells can be sorted when marked with GFP or dsRed (or other fluorescent proteins). The marker protein can either be cytoplasmic, nuclear, or membrane-bound, depending on the choice of the UAS line and the protein localization signals attached to the protein. A large variety of fly strains with UAS-fluorescent-protein transgenes are available from the Bloomington stock center (https://bdsc.indiana.edu/). Figure 7 illustrates the removal of BBB cells from brains in which BBB cells are labeled either by whole cell and membrane-bound expression of GFP, or by nuclear expression of dsRed.

2.4.1.1 Materials

- 50 mg/ml Collagenase A in ddH2O (Millipore Sigma # 10103578001)
- 50 mg/ml DNase I in ddH2O (NEB, # M0303S)
- Schneider's culture medium (BD Biosciences)
- BSA (5 or 10%)
- 0.5 M EDTA
- Thermomixer R (Eppendorf)

- Dissecting forceps
- Eppendorf tubes
- Ice
- 100 um filter unit that fits on top of 50 ml Falcon tube (Falcon Filters, # 352360)

2.4.1.2 Approach

1. Prepare the collagenase solution on the day of use.
2. Pre-heat thermomixer to 37 °C: Fill slots with water, check the temperature with a thermometer
3. Dissect fly brains in cold filtered Schneider's medium containing 1% BSA in batches of 10 to 15 per Eppendorf tube and keep on ice. Dissect for 2 hrs or less.
4. Coat 50 ml Falcon tubes with Schneider's/BSA solution by adding 1 ml to the bottom of the tube, swirl around to coat the bottom and remove. Continue with the same solution to coat all required 50 ml Falcons (1 per sample). Place a 100 μm filter onto the Falcon tube.
5. Pre-coat a 1 ml tip with Schneider's/BSA. Use it to remove the medium from the brain sample by holding the tube up to light to ensure you don't remove the brains from the bottom (be aware of any floating brains).
6. Add 1 ml Schneider's/BSA to wash the samples. Remove solution and replace with 220 μl Schneider's/BSA.
7. Add 10 μl collagenase and 5 μl DNase to the side of the tube and flick gently to mix.
8. Immediately insert tubes into the 37 °C thermomixer and shake at 500 rpm for 5 minutes. Return samples to ice immediately.

9. Add 2.5 µl 0.5 M EDTA to each sample to inactivate the enzymes. Mix, then remove the Schneider's/BSA + brains and add to the appropriate 50 ml Falcon filter. Pipette any drops on the underside of the filter and add to the filtrate (be careful to not add bubbles).
10. Keep the tubes on ice until ready for FACS sorting. We have found it ok to keep cells on ice for transport to FACS sorting facility for 1-2 hrs after dissociation.
11. Prepare one sample with non-labeled BBB cells as a control for FACS sorting.
12. Coat the FACS tube and all pipettes to be used with Schneider's medium/1% BSA. FACS sort the cells with a 100 µm nozzle into Medium/1% BSA or RNA isolation buffer.
13. Non-fluorescent cells are sorted first to determine the window in which they appear. A fair amount of autofluorescence was observed in the control calibration experiment. This control was used to define the window for the collection of GFP positive cells.
14. Samples can be processed for RNA extraction immediately or stored for later use at -80 °C.

2.4.1.3 Notes

- We recommend following the procedures recommended by your FACS core for sorting.
- It is helpful to have a dissecting scope with a UV source for dissection to check progress, but not necessary.

- In our experience, there is a fairly large number of auto-fluorescing cells and some cell debris. We have set a stringent cutoff for fluorescent positive cells.
- Yield from about 30-50 brains has been around 500-700 SPG cells. It is possible that this number can be increased when cells can be sorted sooner after dissociation.
- Collagenase A: We have found that different lots of collagenases can vary widely in their efficiency to dissociate BBB cells (even when ordered from the same supplier under the same order number). Therefore, new batches need to be optimized. Incubate samples at 750 rpm for 5 mins if collagenase is ineffective at 500 rpm for 5 mins. We found one batch that was unable to remove the cells. A comparison of the composition of different lots with the help of the supplier showed that while collagenase amounts were similar, the preparation contains other proteases whose amounts can vary widely. It is possible that they contribute to the dissociation of the cells and the variability among lots.
- If cells are not sorted, but the goal is to just enrich for SPG cells, following dissociation and filtration the cells can be pelleted and dissolved in the desired solution. For example, the cells can be placed on a slide for visualization. If the volume containing the isolated cells is larger than desired, the cells can be pelleted by centrifugation at 4 ^{o}C 5000 rpm for 10 mins and resuspended in the volume and medium of choice.

2.4.2 Remove cells by dissection and forceps

We have found that due to the coherence of BBB cells (due to their septate junctions) it is possible to remove the cells in clusters under a dissecting miscroscope with a UV light

source using fine forceps. These collections will contain some non-BBB cells and will not be as clean as FACS sorted preparations.

2.4.2.1 Materials

- Flies in which SPG cells have been marked by expression of a fluorescent protein such as GFP or dsRed.
- Dissecting microscope with UV light
- Small petri-dish filled half with 1.5% agarose, covered with 1x PBS, for dissection
- Ice
- Forceps: Either ultra-fine (Dumont #5SF Forceps, order# 11252-00) or fine (Dumont#5 fine forceps for dissection, straight, # 11254-20). Both can be used.
- Dry Ice

2.4.2.2 Methods

1. Anesthetize flies on ice
2. Dissect flies on ice in a small petri dish half-filled with agarose covered with cold PBS
3. Dissect fluorescent cells under a stereomicroscope with a UV source
4. To transfer the cells to a solution such as Trizol, we have found it useful to freeze a droplet of a couple of microliters of Trizol in a weigh boat on top of dry ice. Touching of the little 'frozen ball' with the forceps while the cells are still attached to the dissecting forceps causes the cells to "jump over" to the ice droplet. Several batches of cells can be accumulated on one droplet which can subsequently been frozen at -80 ^{o}C for later processing.

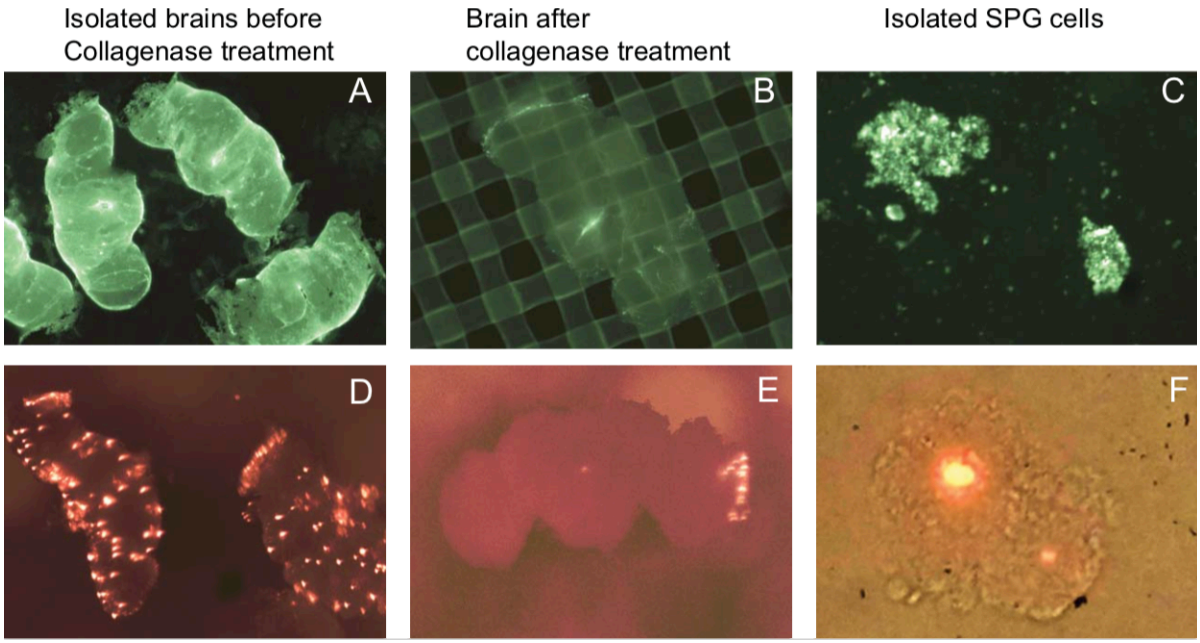


Figure 7: Dissociation of SPG cells from isolated brains

SPG cells were labeled by SPG-specific expression of cytoplasmic GFP (A-C), or by nuclear dsRed (D-F) using the Mdr-Gal4 driver. The whole-cell labeling in (A) illustrates the "cap-like" structure of the BBB. Isolated fly brains are shown (A, D). Following treatment with collagenase, the brains have lost most of the marked SPG cells (B, E). The brain in (B) is situated on the filter that is used to collect dissociated cells. (C): Dissociated GFP labeled SPG cells after sorting. (F) An isolated SPG cell with dsRed expression in the nucleus.

Chapter 3: Materials and Methods

3.1 Brain dissections and immunohistochemistry

The brains of four to five day-old male flies were dissected in 1X Phosphate-buffered saline (PBS) and fixed in freshly prepared 4% paraformaldehyde (1X PBHS buffered: pH 7.4) for 20 min at room temperature.

All subsequent procedures were performed at room temperature, except for antibody incubations.

Brains were washed three times in 1XPBHS/0.5% Triton X-100 for 15 min each, and then washed three times in 0.1M Tris-HCl/0.3M NaCl (pH 7.4), containing 0.5% Triton X-100 (TNT) for 15 min each.

Blocking was performed in TNT solution containing 4% normal goat serum (blocking buffer) for 1.5 hrs. The primary antibody was applied at the proper dilution in blocking solution and incubated overnight at 10 °C. The brains were then rinsed six times for 15 min each and then five times for 30 min each in TNT. The secondary antibody was diluted 1:200 in blocking solutions and incubated overnight at 10 °C. Brains were then washed six times for 15 min each. Fluorescently labeled brains were mounted with Vectashield mounting medium containing DAPI (Vector Laboratories, CA).

For double-staining experiments, antibody stainings were performed sequentially using the procedure described above.

The brains were imaged using a Zeiss SP8 upright confocal microscope.

3.2 Behavioral assays

3.2.1 Courtship Assay

Males were collected immediately following eclosure and each fly was kept in a new food vial for three to five ds before testing their courtship, keeping them socially isolated as they mature. Males are placed on a partially moist filter paper in a courtship chamber (circular arena, 0.8 cm in diameter) with a virgin female collected on the same day as the assay. The courtship index was recorded, which is the fraction of time a male spends performing any steps of courtship toward the female within a 10-min observation period. Equal numbers of controls and mutants were tested in a given session.

3.2.2 Activity Assay

Males were collected immediately following eclosure and each fly was kept in a new food vial for three to five ds before testing their activity. Males are placed on a partially moist filter paper in a courtship chamber and allowed to acclimate to the chamber for two mins before counting the number of times the male crosses an arbitrary line through the center of the chamber within a three-min observation period.

3.3 Conditions for Gal80^{ts} experiment

3.3.1 Knockdown and transgenic expression during adulthood

Fly crosses were kept at 18 ◦C. Males were collected immediately following eclosure and each fly was kept in a new food vial for seven ds at 18 ◦C before half the flies were shifted to 30 ◦C for one to two ds, then shifted to 25 ◦C for one d before testing their courtship at room

temperature (Figure 8). The flies that were not shifted from 18 °C were shifted to 25 °C one d before testing their courtship along with the induced flies.

3.3.2 Knockdown during pupal development and reversal of knockdown in adulthood

The food vials or bottles containing the fly crosses were kept at 25 °C until second instar was reached. Then half the crosses were shifted to 30 °C until eclosure. Freshly eclosed males were collected and transferred into a new vial, then kept for another day at 30 °C before being transferred back to 18 °C for six ds. They were then kept at 25 °C for 1 d before being tested at room temperature (Figure 8A, B, C, D, E). Non-induced flies were kept at 18°C until one d before being tested when they were also transferred to 25°C.

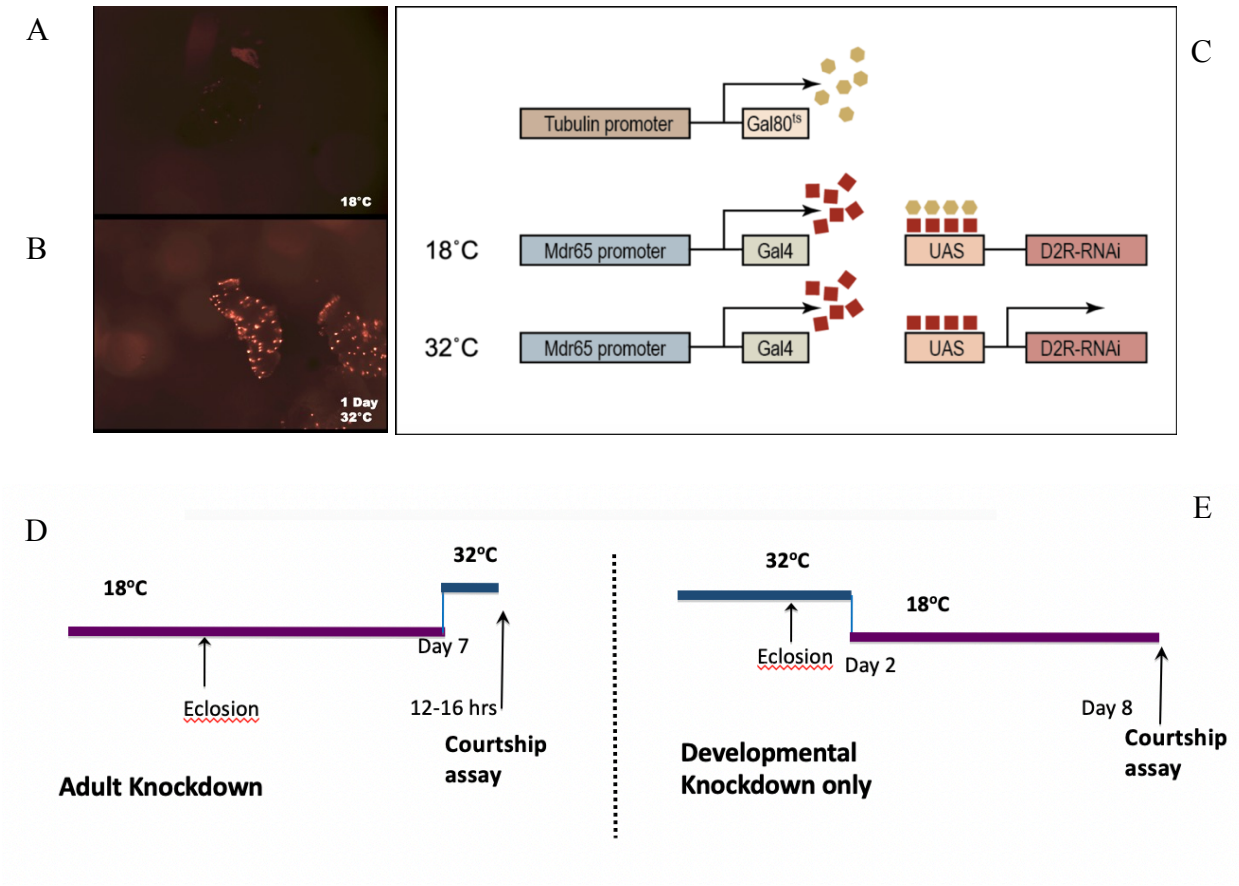


Figure 8: Temperature shift experiment to induce gene expression

In A and B, brains were dissected from flies with the surface glia promoter driving temperature induced expression of dsRed. A) Flies kept at the cold temperature show little to no expression of dsRed. B) Flies kept at the warm temperature for a day show robust dsRed expression in the surface glia. C) Schematic for temperature induced knockdown of D2R. D) For adult knockdown flies are shifted to a higher temperature following eclosure to induce gene expression in the BBB. E) For developmental specific knockdown flies are shifted to a lower temperature following eclosure to block gene expression downstream of the UAS.

3.3.3 Knockdown and transgenic expression immediately following eclosion and during adult maturation

The food vials or bottles containing the fly cross were kept at 18 ◦C and males were collected immediately following eclosure. On d seven, half the flies were shifted to 30 ◦C for two ds and kept at 25 ◦C for one d before testing their courtship at room temperature. The other half were kept at 18 ◦C until they were shifted to 25 ◦C for the day proceeding the courtship assay.

3.4 Protocol for pharmacological experiment

3.4.1 Bromocriptine in fly food

Flies were reared at RT and transferred to Formula 4-24 Instant *Drosophila* Medium Carolina, # 173200, containing DMSO or DMSO with bromocriptine one day before the courtship assay.

3.5 Fly stocks

w^{1118} *PBac{WH}Dop2R*^{f06521} (BL# 85250) D2R hypomorphic mutants were outcrossed for 10 generations with w^{1118}(CS) flies (w^{1118} in a Cantonized background). The w^{1118} allele was exchanged for w+ in both outcrossed D2R hypomorph and null mutants by recombination, since D2R is on the X chromosome.

3.6 Data analysis

Statistical analysis was performed using XLSTAT. Two-way ANOVA followed by Fisher's PLSD was used to assess significant interaction in courtship assays between genotypes and conditions.

Workflow for RNA sequencing analysis included the use of Bowtie for aligning reads to large genomes, TopHat to map splice junctions, Cufflinks to assemble transcripts and estimate expression in samples and SAMtools to identify Single Nucleotide Polymorphisms (SNPs).

Chapter 4: Results

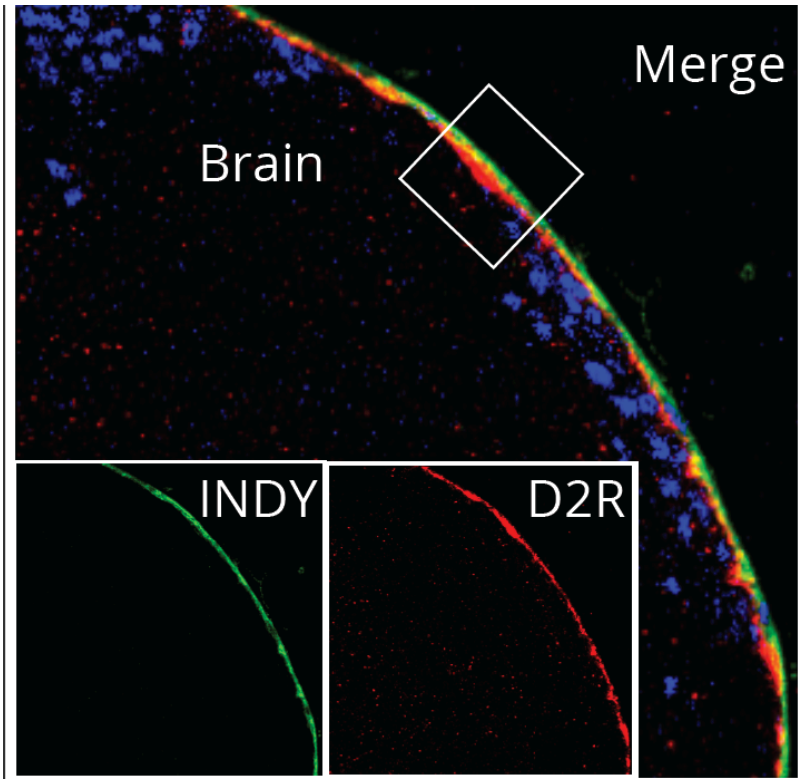
Introduction

The blood-brain barrier of *Drosophila melanogaster* ensheathes the brain in a tight monolayer of polyploid cells called the subperineural glia (SPG). We have previously found that sex-specific factors and GPCR-mediated signaling is required in the adult BBB for normal courtship (Hoxha et al., 2013). Subsequent microarray analysis of male and female BBB cells in the lab identified many sex-preferentially expressed transcripts (unpublished). One of them was for the *Drosophila Dopamine 2-like Receptor (D2R)*. Here, I characterize expression of D2R in the SPG cells of the BBB and examine the role of *D2R* in the BBB in courtship control. I use mutants and the Gal4-UAS system to temporally and spatially reduce RNA levels of the *Drosophila Dopamine 2-like Receptor (D2R)* using RNAi, and to restore levels of D2R in the BBB of *D2R* mutants. These experiments examine the hypothesis that D2R in the BBB has an adult specific role in courtship that is separable from any developmental requirements.

4.1 D2R is expressed in the SPG cells of the BBB

To characterize the presence of D2R in the BBB, I performed immunohistochemsitry with a previously developed antibody (Draper et al., 2007). Fly brains expressing the BBB marker Indy-GFP were stained with antibodies against GFP and D2R. *Indy-GFP* flies carry an insertion of GFP inside the *indy* gene (Quinones-Coello, 2007). The resulting GFP expression marks both layers of the BBB (PG cells and SPG cells). Double-staining of *Indy-GFP* brains with anti-GFP and with anti-D2R showed that Indy-GFP and D2R partially co-localize on the basal side of the BBB (Figure 9c). This suggests that D2R is expressed specifically in the SPG (Figure 9). To confirm that indy-GFP indeed expresses in both SPG and PG cells, flies

were created that carry the SPG specific *Mdr-Gal4*, *UAS-mcd8-RFP* and *Indy-GFP*. Figure 9 shows the SPG-specific expression of RFP, and the expression of Indy-GFP in both SPG and PG cells (Figure 9a-f). These results suggest the expression of D2R in the SPG cells of the BBB.



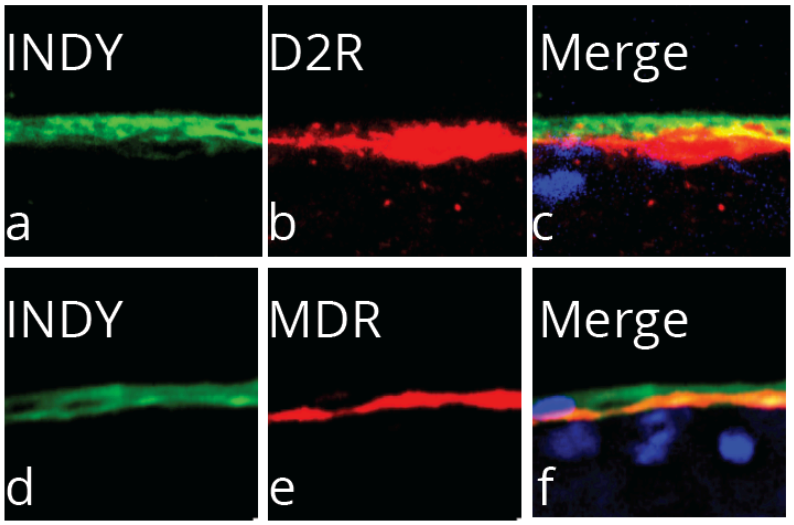


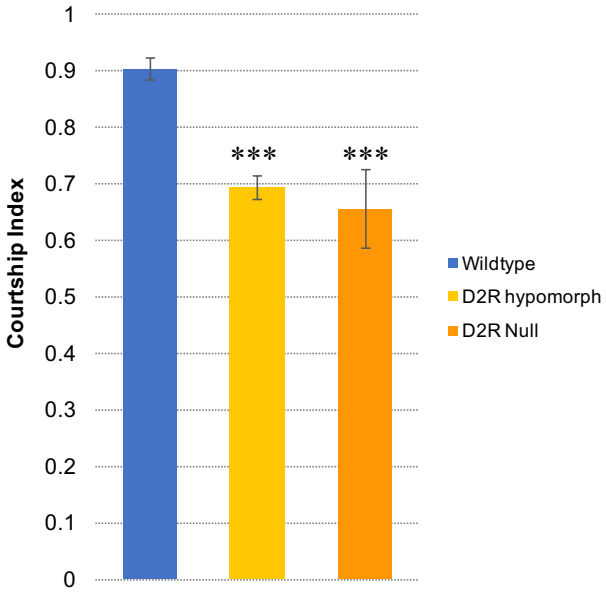
Figure 9: D2R is expressed in the SPG cells of the BBB

Fly brains expressing the BBB marker Indy-GFP that marks both PG and SPG cells were stained with antibodies against GFP and D2R (Draper et al., Dev Neurobiology 2007). Expression of Indy-GFP and D2R co-localized in the SPG (a-c). d-f: Mdr-Gal4 driven expression of dsRed marks SPG cells (e, f, red, and yellow, respectively), whereas Indy-GFP marks both PG (top) and SPG cells (d, f, green, and yellow, respectively). Blue: DAPI staining of neuronal nuclei.

4.2 D2R mutants have courtship defects

To determine if D2R in the BBB plays a role in courtship, D2R mutants were behaviorally tested using courtship and activity indices. D2R hypomorphic and null mutants have reduced courtship indices compared to wildtype controls (Two way ANOVA with post-hoc Bonferroni multiple comparisons test; ***, $p < .0001$). Short-term activity levels are not different

between wildtype controls and D2R mutants (Figure 10). All flies were reared at room temperature and tested on day-4 post-eclosure.



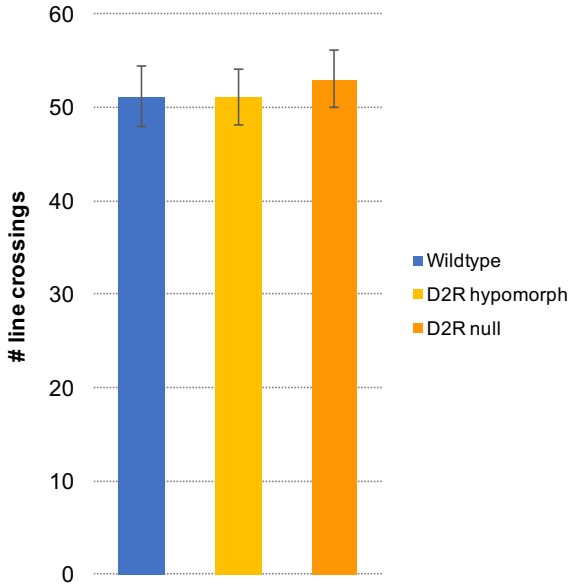


Figure 10: D2R mutants have a courtship defect. Genotypes are color coded. The wildtypes in blue have normal levels of courtship, the Hypomorphic mutants in yellow and Null mutants in orange have reduced courtship. The number of line crossings between genotypes is consistent indicating normal locomotion. Error bars denote S.E.M. n=20

4.3 D2R splice variants: enrichment of D2R-RH long isoform

To identify the predominant isoforms of D2R and expression levels of major players in D2R signaling and BBB function, I examined mRNA expression levels of individual isoforms in whole brains by RNA-seq analysis using TopHat (Figure 11). *pale* (*ple*) encodes tyrosine hydroxylase, the rate limiting enzyme for dopamine synthesis. The *ple* gene is expressed exclusively in dopaminergic neurons and includes three isoforms with one major form. *Fruitless* (*fru*) is a behavioral sex determination gene that produces 11 splice variants in the *fru* neurons with one major form and several minor forms. The *Drosophila* nonvisual arrestin *kurtz* (*krz*) is a regulator of GPCR activity and contains one major form. *Moody* is an SPG specific GPCR with several splice variants including two major forms that have courtship and cocaine response roles and are required for the tight barrier formation and maintenance of the BBB. *Mdr65* encodes one isoform that is expressed specifically in the SPG at a high level. The *D2R* locus in *Drosophila* contains six isoforms with two major variants, long and short. My analysis identifies the RH long isoform as the preferentially expressed isoform in the brain, with the short form expressed at lower levels.

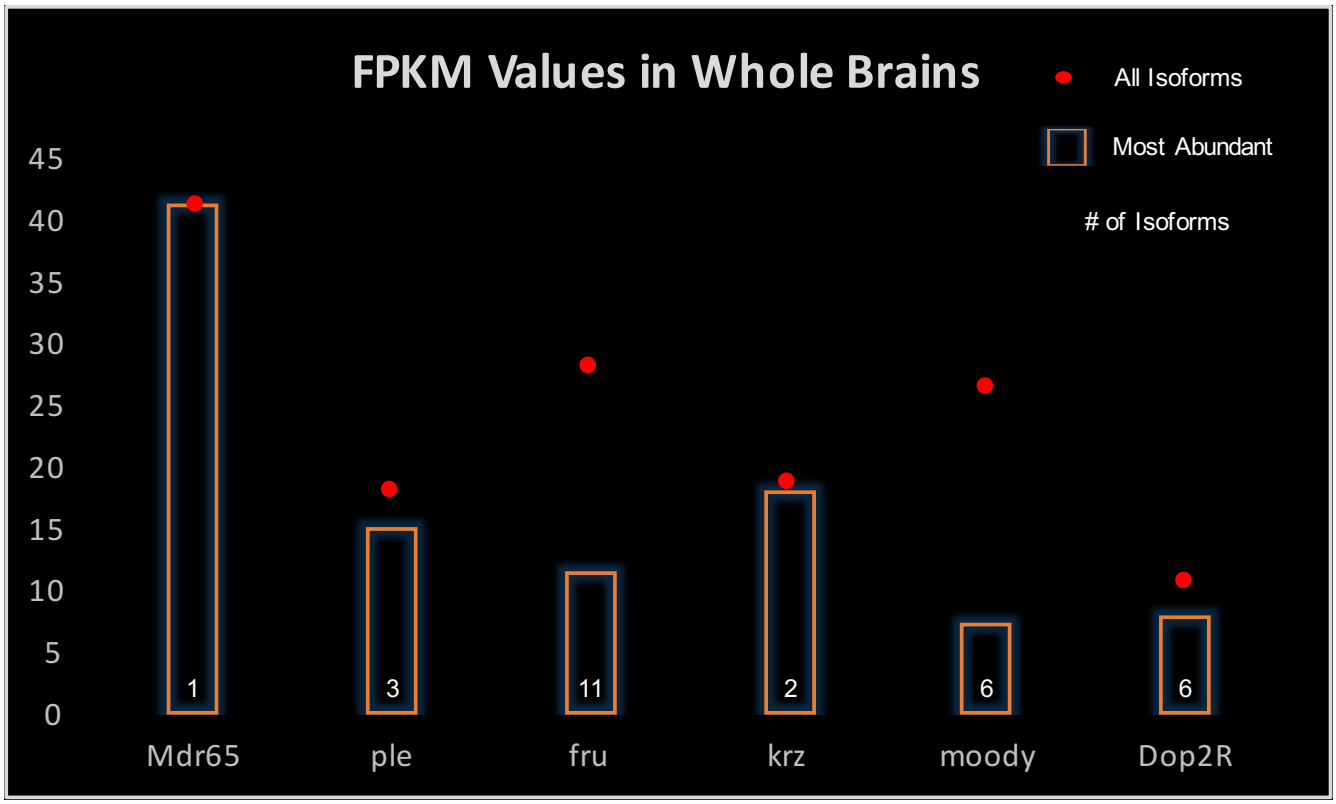


Figure 11: D2R Splice Variants and abundance from RNA sequencing.

Expression levels of various isoforms for genes in *Drosophila* whole brains. The expression of *Mdr65* is high in relation to *D2R* expression. The D2R RH isoform is the most abundant isoform. Numbers inside the columns represent total number of isoforms.

4.4 Induced adult expression of D2R in the BBB rescues the courtship defects of mutants

To determine whether the predominant isoform of D2R can rescue the courtship defects in *D2R* mutants, I generated *w+, D2R Null* flies with *Mdr-Gal4* and *tubulin-Gal80^{ts}* transgenes and crossed them with transgenic flies carrying *UAS-D2R RH* long form. D2R –RH was synthesized and inserted into the *pUAST* transformation vector to generated *UAS-D2R RH* transgenic flies. At d seven, progeny were shifted from 18 °C to 30 °C for one d. Flies from

18 °C and 30 °C were shifted to 25 °C for 1 d before the courtship assay. Induction of *D2R* in adults rescues the courtship index in *D2R* mutants (Two way ANOVA with post-hoc Bonferroni multiple comparisons test; ***, $p < .0001$). Controls have normal levels of courtship and mutants without the *UAS-D2R-RH* rescue construct have reduced courtship. Temperature conditions do not significantly change the index in controls or mutants (Figure 12).

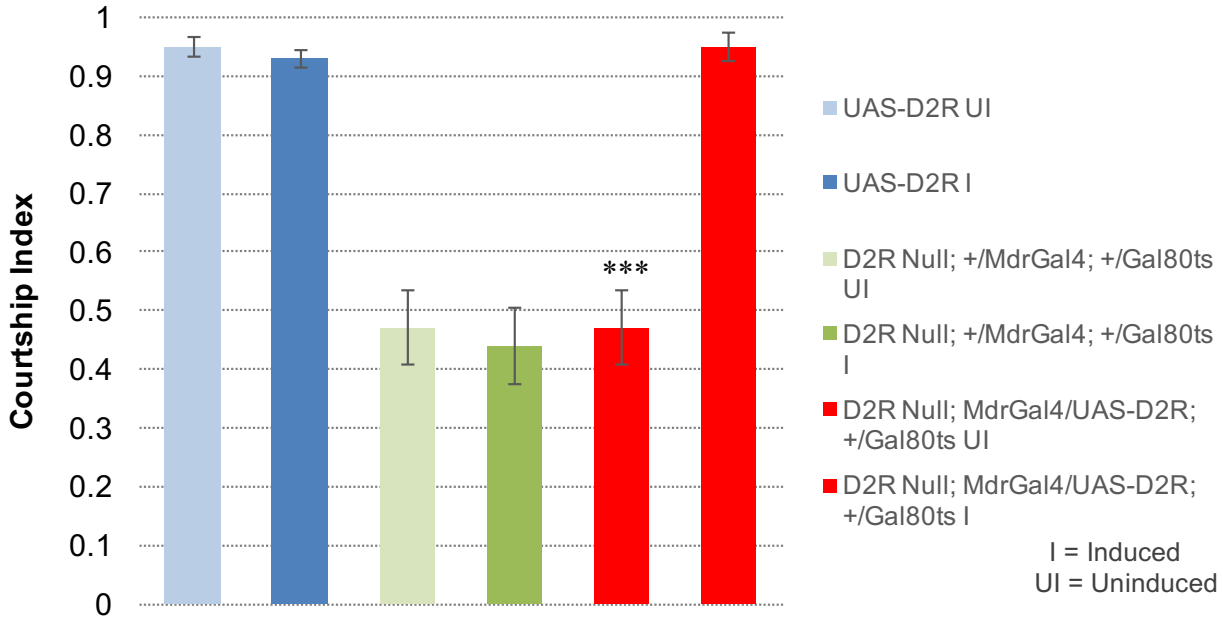


Figure 12: Induced adult expression of D2R in the BBB rescues the courtship defects of mutants. Genotypes are color coded. *Mdr-Gal4* is SPG specific (Schwabe, 2005). Genetic controls are each heterozygous for one component of their respective binary Gal4-UAS. Induced adult D2R expression in the BBB rescues the courtship index in *D2R* null mutants. Uninduced *D2R* mutant flies do not show courtship rescue. Induced and non-induced flies with just *UAS-D2R* have full courtship indices. Error bars denote S.E.M. n=20

4.5 Expression of D2R-RNAi in the BBB of mature adult males reduces courtship

To test whether knockdown of D2R in the adult BBB reduces courtship, I crossed *Mdr-Gal4; tubulin- Gal80^{ts}* flies to *UAS-D2R-RNAi* flies to reduce *D2R* by expressing the RNAi in the adult BBB. Induced flies were shifted from 18 °C to 30 °C for one d before being shifted for the courtship assay. Flies from 18 °C and 30 °C were shifted to 25 °C for one d before the courtship assay. The knockdown of *D2R* in the SPG reduces the courtship index (Two way ANOVA with post-hoc Bonferroni multiple comparisons test; ***, $p < .0001$). The courtship index for controls is normal and consistent among temperature conditions. Activity levels of controls and *UAS-D2R RNAi* induced flies are the same (Figure 13).

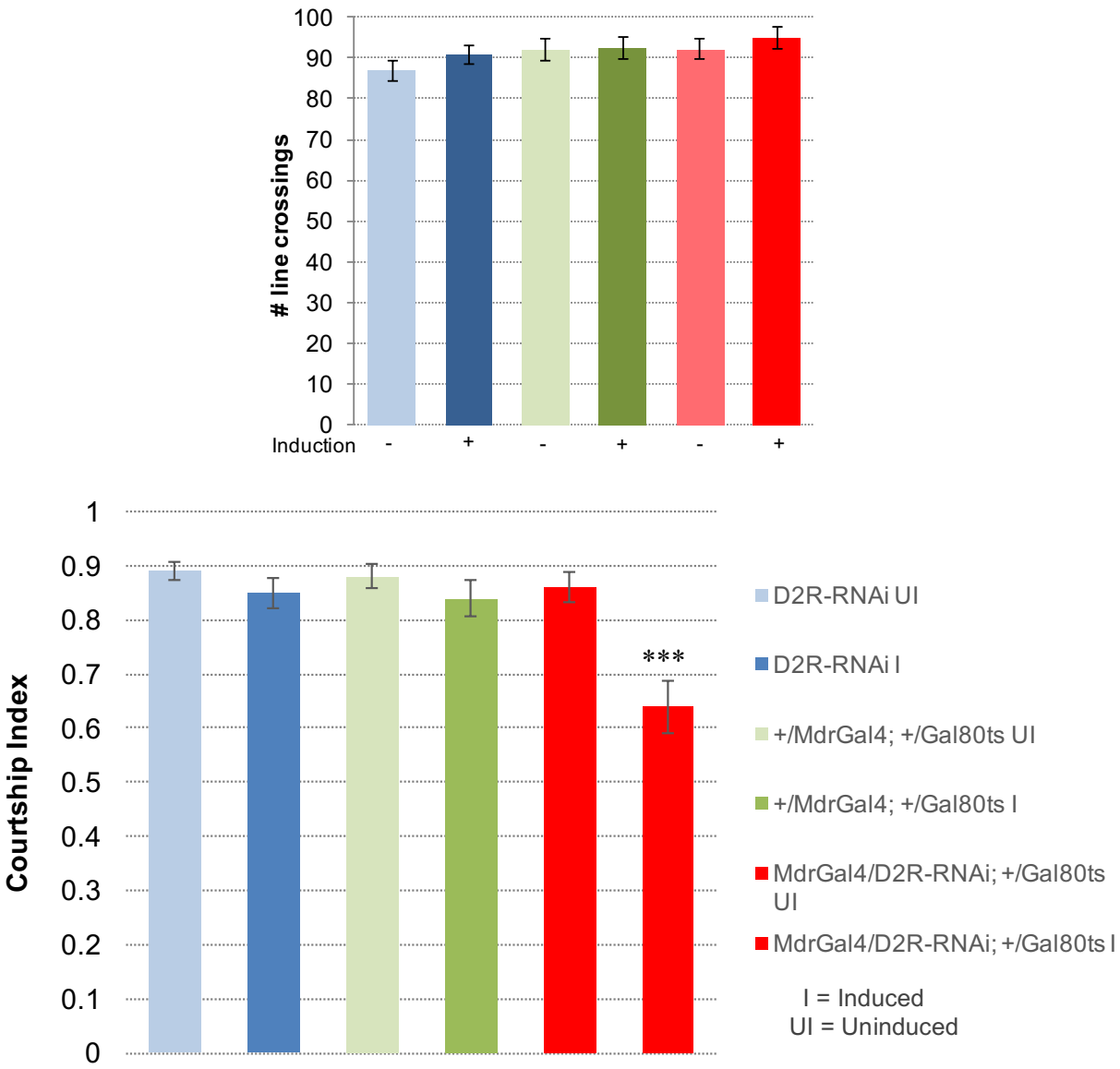


Figure 13: Expression of D2R-RNAi in the BBB of mature adult males reduces courtship. Genotypes are color coded and temperature conditions are marked uninduced (UI) or induced (I). Genetic controls are each heterozygous for one component of their respective binary Gal4-UAS. D2R RNAi knockdown in the SPG of mature adults reduces courtship. Knockdown does not affect general locomotion. Error bars denote S.E.M., n=20

4.6 Knockdown of D2R in the BBB during pupal development does not reduce courtship

To identify if D2R has a role in the BBB for male courtship that is required during development, I crossed *Mdr-Gal4; tubulin-Gal80^{ts}* flies to *UAS-D2R-RNAi* flies to reduce D2R by expressing the RNAi in the larval BBB. Once second instar was reached, induced flies were shifted from RT to 30 ◦C until maturation then half were moved to 18 ◦C for six ds before being shifted for the courtship assay. Flies from 18 ◦C and 30 ◦C were shifted to 25 ◦C for one d before the courtship assay. The knockdown of D2R in the SPG reduces the courtship index (Two way ANOVA with post-hoc Bonferroni multiple comparisons test; ***, $p < .0001$), but down-shifting (i.e., ending *D2R*-RNAi expression) to 18 ◦C upon eclosing fully rescues the courtship index. These data support a physiological requirement for D2R specifically in adult flies. The courtship index for controls is normal and consistent among temperature conditions (Figure 14).

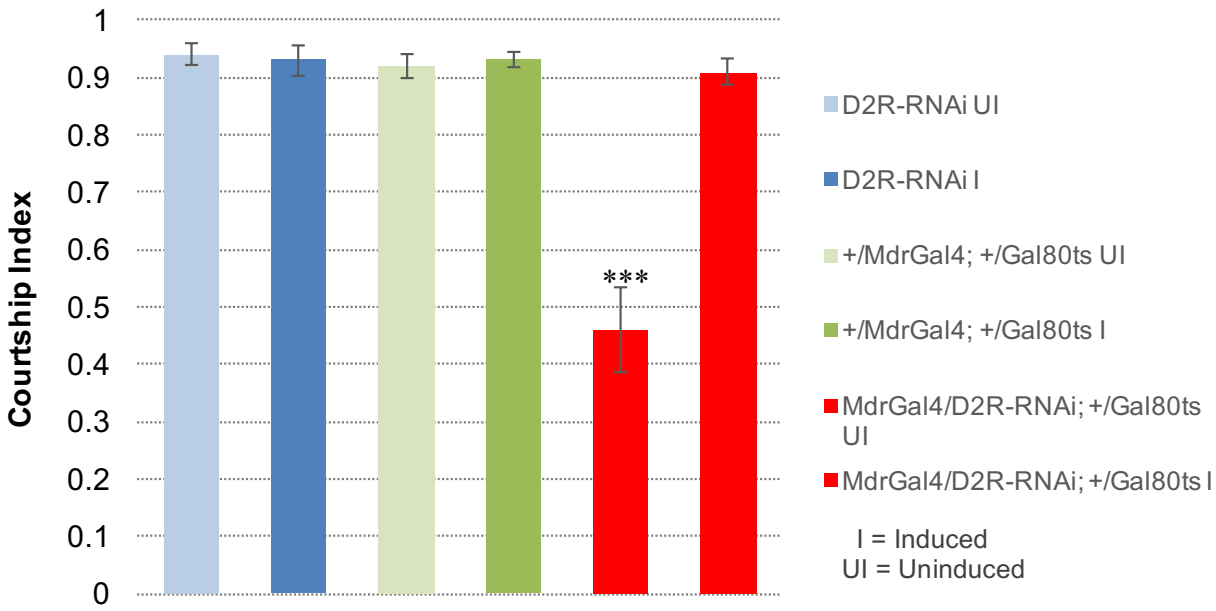


Figure 14: Knockdown of D2R in the BBB during pupal development does not reduce courtship. Genotypes are color coded and RNAi expression conditions are marked uninhibited (UI) and inhibited (I). Courtship reduction from D2R knockdown in SPG from 2nd instar to mature adult is fully recovered by RNAi suppression in mature adults. Error bas denote S.E.M., n=20

4.7 Disruption of D2R in the BBB does not compromise its barrier function

To probe BBB integrity in the face of D2R disruption, D2R hypomorphic mutants and flies with D2R-RNAi knockdown in the SPG were injected with 10 kD Texas Red-Dextran, allowed to recover for one d and dissected in fixative to test for dye penetration into the brain. The exclusion barrier in D2R mutants and knockdown flies is not compromised (Figure 15).

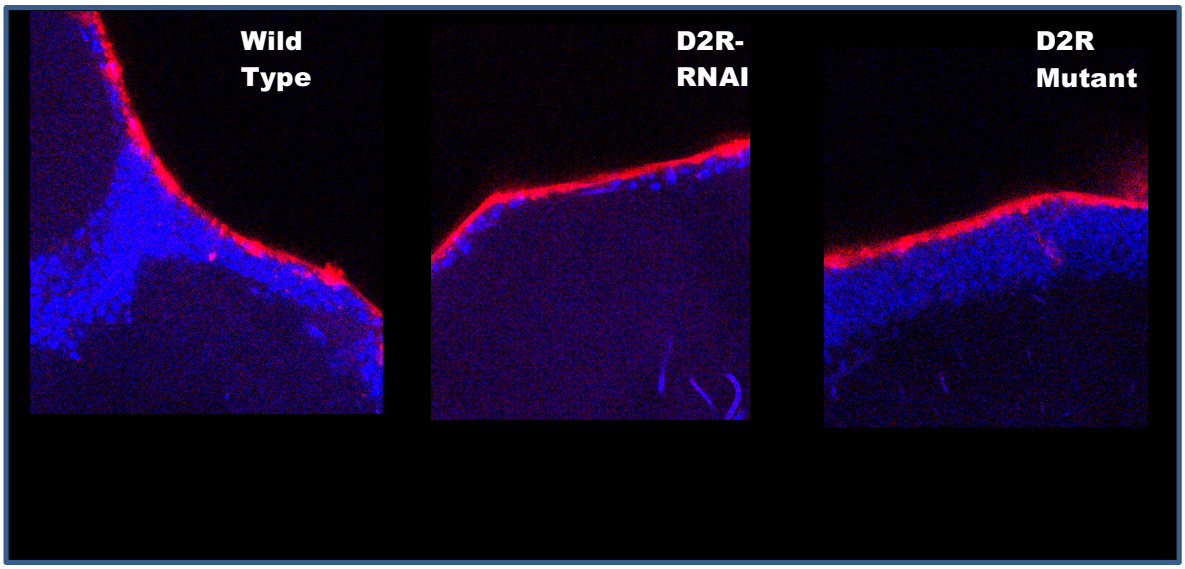


Figure 15: Disruption of *D2R* in the BBB does not compromise its barrier function. 10 kD Texas Red-Dextran accumulates at the BBB in D2R Mutants or D2R-RNAi knockdown flies and does not penetrate the brain(red). Neuronal nuclei are stained with Dapi (blue).

4.8 D2R knockdown in neurons does not reduce courtship

To identify if D2R is also required in neurons for full levels of courtship, I crossed *elav-Gal4* (a pan-neuronal driver) with *UAS-D2R-RNAi* flies to generate flies with D2R knockdown in all neurons. Courtship is not reduced by *D2R* knockdown in neurons. The courtship index is consistent among controls and knockdown. All flies were reared at room temperature and tested on day-four post-eclosure (Figure 16)

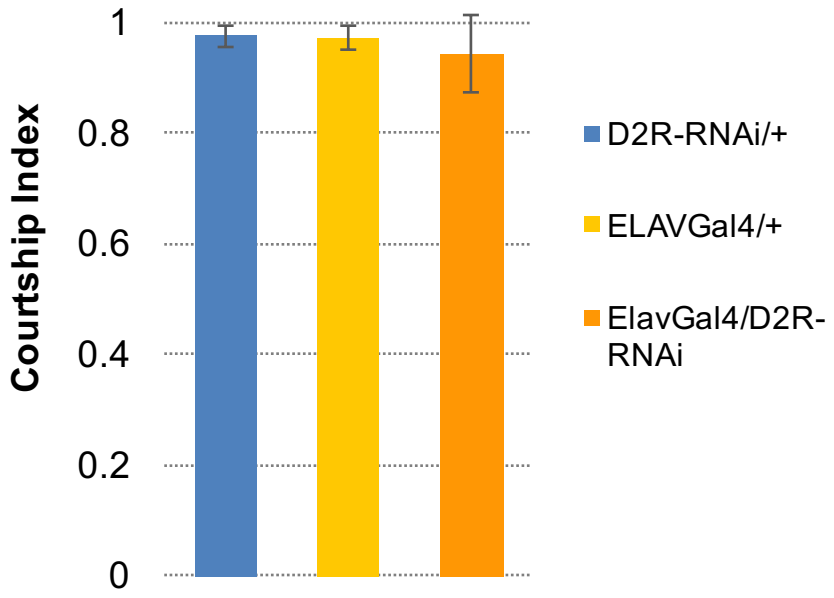


Figure 16: D2R knockdown in neurons does not reduce courtship Genotypes are color coded. Genetic controls are each heterozygous for one component of their respective binary Gal4-UAS. D2R RNAi knockdown in neurons does not reduce courtship. Error bars denote S.E.M, n=20

4.9 Over-expression of D2R in the BBB reduces courtship

As shown above, reduction of *D2R* in the BBB reduces courtship. To examine whether *D2R* overexpression in the BBB or in neurons affects courtship, *nsyb-Gal4* (a pan-neuronal driver) was crossed with *UAS-D2R RH* to overexpress *D2R* in neurons, and *Mdr-Gal4* was crossed with *UAS-D2R RH* to overexpress *D2R* in the SPG. Courtship is not reduced by overexpressing *D2R* in neurons. In contrast, we found that overexpression of *D2R* in the SPG reduces courtship (Two way ANOVA with post-hoc Bonferroni multiple comparisons test;

***, p≤.0001). This suggests a requirement for D2R levels within an optimal range for courtship. The courtship index is normal in controls. All flies were reared at room temperature and tested on day-four post-eclosure (Figure 17).

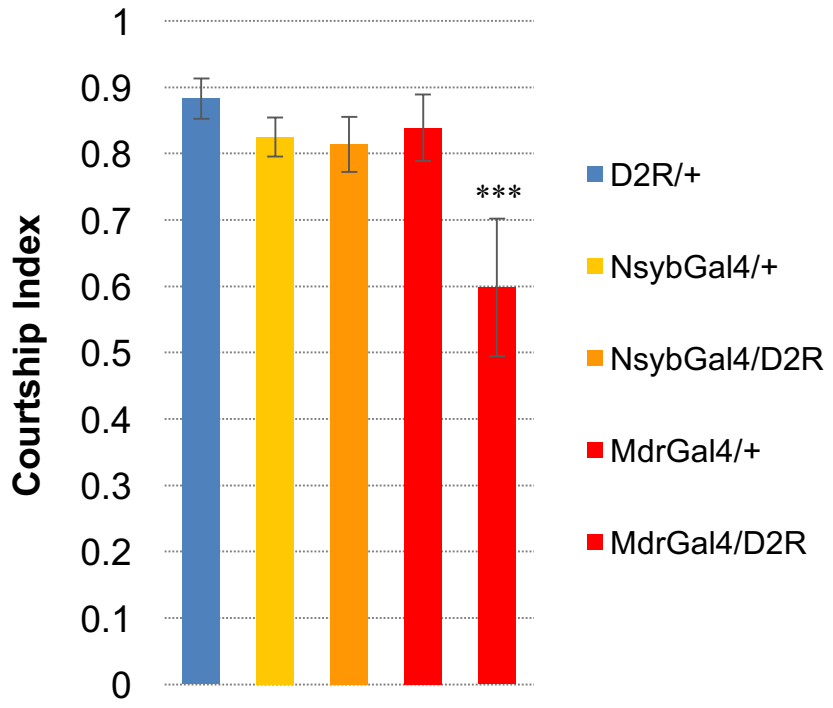


Figure 17: Over-expression of D2R in the BBB reduces courtship Genotypes are color coded. Genetic controls are each heterozygous for one component of their respective binary Gal4-UAS. D2R overexpression in the SPG reduces courtship. Expression of D2R in neurons does not reduce courtship. Error bars denote S.E.M., n=20

4.10 D2R hypomorphic but not null mutants have normal levels of courtship by day 7 post-eclosure

To examine if courtship indices recover with time in *D2R* mutants, *D2R* hypomorphic, *D2R* null and CantonS (CS) flies were tested for courtship either at d four post eclosure or d seven at 25 °C. The courtship index for *D2R* null flies was reduced at both four and seven ds of age (Two way ANOVA with post-hoc Bonferroni multiple comparisons test; ***, $p < .0001$). In contrast, the courtship index for *D2R* hypomorphs was reduced at d four (Two way ANOVA with post-hoc Bonferroni multiple comparisons test; ***, $p < .0001$) and partially recovered by d seven (Two way ANOVA with post-hoc Bonferroni multiple comparisons test; *, $p < .01$). The courtship index for CS controls is normal under both conditons (Figure 19). This suggests that sufficient amounts of D2R can accumulate in hypomorphs over time to allow for normal courtship.

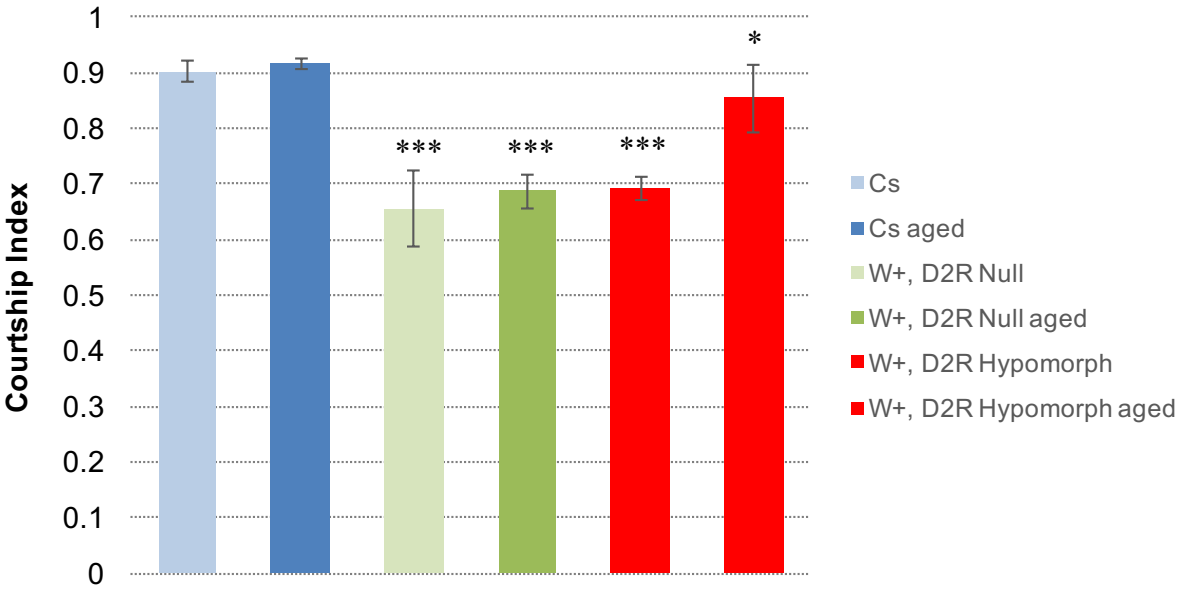


Figure 18: D2R hypomorphic mutants have normal levels of courtship by day 7 post-eclosure. Genotypes are color coded. Aged D2R hypomorphic mutants recover their courtship. Error bars denote S.E.M., n=20

4.11 Adult BBB knockdown of β-arrestin reduces courtship in sensitized mutant

D2R is a G-protein-coupled receptor whose action is likely mediated by beta-arrestin. To examine if the GPCR coupled signaling molecule beta-arrestin is required in the BBB for male courtship *Mdrgal4; tubulin-Gal80^{ts}* flies were crossed with *UAS krz-RNAi* in flies that are also heterozygous for a *krz* null mutation, thus testing beta-arrestin knockdown in a sensitized background. krz is the nonvisual *Drosophila* homolog of beta-arrestin. Induced flies were shifted from 18 ◦C to 30 ◦C for one d before being shifted for the courtship assay.

Flies from 18 °C and 30 °C were shifted to 25 °C for one d before the courtship assay. While heterozygous *krz* mutants court normally, additional knockdown of *krz* in the SPG of sensitized (heterozygous) mutant flies reduces the courtship index (Two way ANOVA with post-hoc Bonferroni multiple comparisons test; ***, $p < .0001$). Courtship index for controls and sensitized mutants is normal and consistent among temperature conditions (Figure 18).

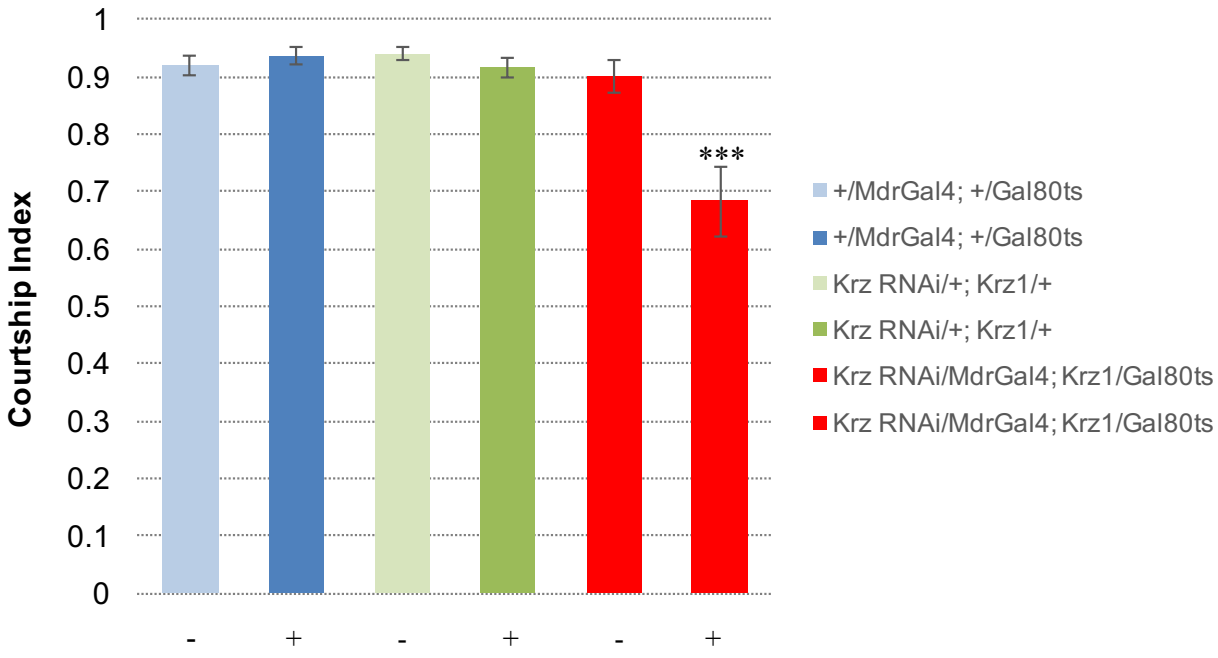


Figure 19: Adult BBB knockdown of β-arrestin reduces courtship in a sensitized mutant. Genotypes are color coded and temperature conditions are marked uninduced (-) or induced (+). Genetic controls are each heterozygous for one component of their respective binary Gal4-UAS. Adult knockdown of β-arrestin in the BBB of heterozygous mutants reduces courtship. Error bars denote S.E.M., n=20

4.12 Adult BBB knockdown of β-arrestin in *D2R* hypomorphs extends courtship reduction

To test potential genetic interactions between *D2R* and beta-arrestin, *D2R* null or *D2R* hypomorphs with *Mdr-Gal4; tubulin-Gal80^{ts}* were crossed with *UAS-krz-RNAi* to reduce *krz*-encoded beta-arrestin expression in the BBB of adult mutants with reduced or missing expression of *D2R*. *Mdr-Gal4; Gal8^{ts}, UAS-krz-RNAi* flies have normal courtship. We predict that if *D2R* and *krz* interact genetically, we will observe courtship levels in the double mutants that are lower than in the *D2R* mutants alone, indicating enetic interaction. Induced flies were shifted from 18 ◦C to 30 ◦C for one day before being shifted for the courtship assay. Flies from 18 ◦C and 30 ◦C were shifted to 25 ◦C for one d before the courtship assay. The courtship index is reduced and consistent in *D2R* nulls with or without *krz* knockdown. The index is reduced in uninduced hypomorphs without *krz* knockdown and slightly higher in induced hypomorphs without *krz* knockdown (a possible age effect). Uninduced D2R hypomorphs with inhibited krz RNAi have slightly reduced courtship and induced D2R hypomorphs with *krz* RNAi knockdown have a severely reduced courtship index, indicating that D2R and beta arrestin act in the same pathway that is needed for normal courtship. Controls have a normal courtship index among temperature conditions (Figure 20).

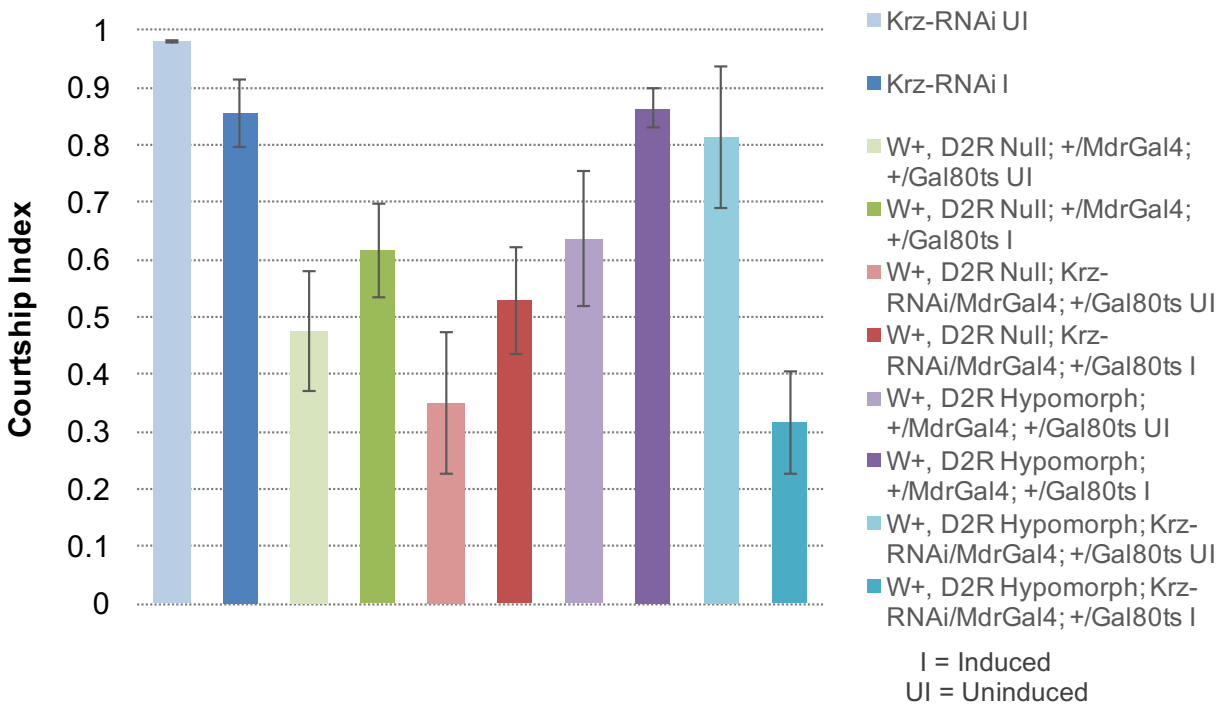


Figure 20: Adult BBB knockdown of β-arrestin in D2R hypomorphs extends duration of courtship reduction. Genotypes are color coded and temperature conditions are marked uninduced (UI) or induced (I). Genetic controls are each heterozygous for one component of their respective binary Gal4-UAS. Adult knockdown of β-arrestin in the BBB of D2R hypomorphic mutants reduces courtship. Error bars denote S.E.M. what is the n=10

4.13 Bromocriptine rescues D2R hypomorphic mutants

To test if D2R ligand induced activation rescues the courtship defects in mutants, *D2R* hypomorphic, null and control flies were treated with the D2R agonist Bromocriptine, to stimulate D2R activity in adult flies with reduced or missing D2R. Flies were reared at RT and shifted to food with bromocriptine or vehicle one d before the courtship assay. *D2R* null

flies have a reduced courtship index with or without bromocriptine treatment (Two way ANOVA with post-hoc Bonferroni multiple comparisons test; ***, $p < .0001$). The courtship index in *D2R* hypomorphic flies without bromocriptine is reduced and the index recovers with bromocriptine treatment (Two way ANOVA with post-hoc Bonferroni multiple comparisons test; **, $p < .001$). The courtship index is normal in controls with or without bromocriptine treatment (Figure 21).

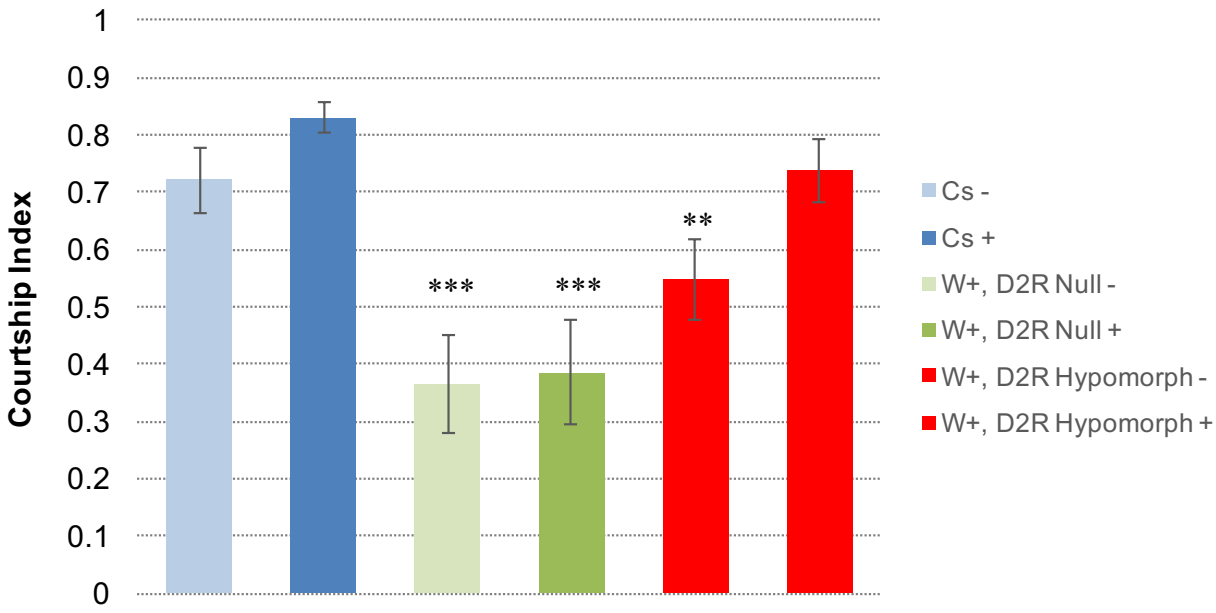


Figure 21: Bromocriptine rescues D2R hypomorphic mutants. Genotypes are color coded and flies fed with vehicle are marked (-) and flies fed with vehicle and drug are marked (+). D2R hypomorphic mutants treated with Bromocriptine show increased courtship. Error bars denote S.E.M.

Chapter 5: Discussion

5.1 Conclusion

Dopaminergic systems are known to modulate various behaviors, including locomotor activity and courtship. The roles of dopamine and its receptors in behaviors have been described in multiple neuronal circuit analyses. Here we show that the glial cells that form the blood-brain barrier (BBB) modulate male courtship behavior through a dopamine receptor in *Drosophila*. We have identified the *Dopamine 2- like Receptor (D2R)* in the subperineurial glia (SPG) in a BBB microarray screen and confirmed its presence using a D2R antibody. The action of D2R and β-arrestin in the BBB is important for sustaining courtship attention. The levels and timing of expression in the organism is important for the behavior.

• Conditional knockdown of *D2R* in the BBB of adult mature males reduces courtship.

• Conditional knockdown of *krz* in the BBB of sensitized *krz* mutants reduces courtship.

• Continuous knockdown of *D2R* reduces courtship and is fully restored with suppression of the RNAi in mature adults.

• A *D2R* mutant shows similar courtship defects that can be rescued by induced *D2R* expression in the BBB of mature adults.

• Overexpression of *D2R* in the adult BBB reduces courtship.

• Overexpression or knockdown of *D2R* in neurons has no effect on courtship.

• *krz* knockdown specifically in *D2R* hypomorphs reduces courtship.

• The *D2R* agonist bromocriptine rescues courtship specifically in *D2R* hypomorphs.

• These results indicate a signaling role for *D2R* in the adult BBB that is important for male courtship behavior.

Astrocytes are hyperpolarized by dopamine stimulated activation of dopamine receptors on glial cells (Wiemerslage et al., 2013). D2 receptors are strongly expressed in astrocytic processes surrounding cortical interneurons in the prefrontal cortex (Hosli et al., 1987). D2 receptor stimulation also induces Ca2+ elevation in astrocytes (Zafar et al., 2001; Bal et al., 2001; Cooper et al., 1995). Intracellular Ca2+ oscillations and intercellular Ca2+ waves in glia may generate changes in glial cell physiology that potentially alter the excitability of neuronal networks via regulation by D2R activity. Gap junction proteins in the BBB control reactivation of neural stem cells by enabling the glia to respond to nutritional signals from the fat body and induce calcium oscillations in the glial cells that result in changes to the stem cells in the brain via activation of insulin receptors from insulin produced in the BBB (Spéder et al., 2014). D2R in the BBB may act in a similar manner to relay signals from dopaminergic neurons to regulate cellular activity and the production of adult and sex-specific behaviors.

Mechanisms of circadian regulation in the BBB may also play into the regulation and feedback of these signaling pathways in the BBB cells (Balaji et al., 2017; Zhang et al., 2018).

5.1.1 Conserved sites in *Drosophila* D2R for site specific action

The *Drosophila* Dopamine 2-like Receptor has over 80% conservation of amino acids compared to the mammalian D2R at functionally conserved sites (Figure 22).

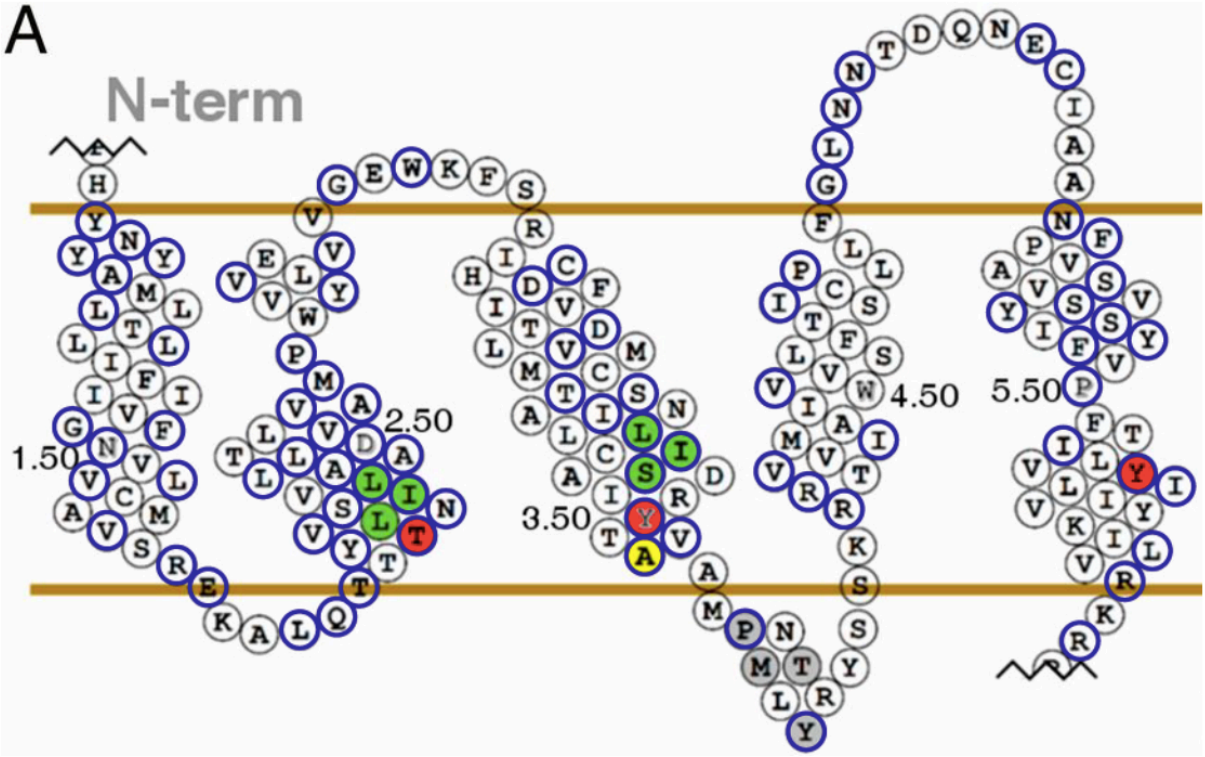


Figure 22: D2R residues snake-plot. Conserved sites between *Drosophila* and mammals are marked in blue. Green and yellow indicate Gα mediated biased activity sites. Red and gray indicate Beta-Arrestin mediated biased activity sites.

5.1.1.1 Differential signaling role for D2R through Go and β-arrestin specific pathways

Dopamine receptors belong to the superfamily of G protein-coupled receptors (GPCRs) that produce the majority of their effects by binding to heterotrimeric G proteins which consist of an alpha subunit, and a dimeric gamma and beta subunit. There are 5 subtypes of the Dopamine receptors in humans, D_1 – D_5. The receptors fall into two families: the D_1 and D_5 receptors stimulate adenylyl cyclase to produce cAMP, and Dopamine D_2, D_3, and D_4 receptors that produce the opposite effect and inhibit the activity of adenylyl cyclase and the amplification of PKA phosphorylation activity. This occurs through the activity of their respective $G_{\alpha s}$ and $G_{\alpha i/o}$ when following the binding of dopamine, or in other cases as a result of constitutive receptor activity, perhaps as a consequence of GPCR heteromer networks (Beaulieu et al., 2015). These processes go on to activate specific signaling pathways (Kienast and Heinz, 2006; Le Foll et al., 2009). Dopamine receptors also activate cAMP-independent pathways, including the recruitment of beta arrestin that acts to desensitize the receptors and also initiates its own signaling pathway leading to activation of extracellular signal-regulated kinases (Beaulieu and Gainetdinov, 2011). Dopamine receptors are implicated in numerous

neurological processes and modulation of neuroendocrine signaling. Dysfunction of dopaminergic neurotransmission in the CNS is implicated in a variety of neuropsychiatric disorders, including Parkinson's disease, ADHD, schizophrenia, and drug and alcohol dependence. Synthetic agonists and antagonists for dopamine receptors are often used to treat these disorders (Mustard et al., 2005).

The development of functionally selective drugs is opening a pathway to a new generation of antipsychotics that target D2R with reduced side effect profiles for the treatment of schizophrenia (Kenakin and Christopoulos, 2013). Antipsychotic drugs can antagonize both G-protein and G-protein independent signaling by binding D2R and stabilizing different structural conformations (Weiwer et al., 2018; Wang et al., 2018; Sathyamangla., 2017; Luttrell and Luttrel, 2003). Biased ligands have been discovered that induce selective confirmations in the receptor that bias it toward G-protein or G-protein independent pathways depending on the ligand. In addition to the discovery of biased ligands, functionally selective D2R mutants that are biased towards G-protein or G-protein independent pathways can be generated to identify pathway functions and model *in vivo* therapies (Wootten et al., 2018; Hilger et al., 2018; Peterson et al., 2015; Peterson et al., 2015). Genetic tools such as Designer Receptors Exclusively Activated by Designer Drugs (DREADD) and trans-Tango can be utilized to identify D2R selective functions in *Drosophila* and elucidate their importance for adult and sex-specific behaviors. (Becnel et al., 2013; Roth, 2016; Talay et al., 2017).

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