Significance of DopEcR, a G-protein Coupled Dopamine/Ecdysteroid Receptor, in Physiological and Behavioral Response to Stressors

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Abstract

Organisms respond to various environmental stressors by modulating physiology and behavior to maintain homeostasis. Steroids and catecholamines are involved in the highly conserved signaling pathways crucial for mounting molecular and cellular events that ensure immediate or long-term survival under stress conditions. The insect dopamine/ecdysteroid receptor (DopEcR) is a dual G-protein coupled receptor for the catecholamine dopamine and the steroid hormone ecdysone. DopEcR acts in a ligand-dependent manner, mediating dopaminergic signaling and unconventional "nongenomic" ecdysteroid actions through various intracellular signaling pathways. This unique feature of DopEcR raises the interesting possibility that DopEcR may serve as an integrative hub for complex molecular cascades activated under stress conditions. Here, we review previously published studies of *Drosophila DopEcR* in the context of stress response and also present newly discovered *DopEcR* loss-of-function phenotypes under different stress conditions. These findings provide corroborating evidence that DopEcR plays vital roles in responses to various stressors, including heat, starvation, alcohol, courtship rejection, and repeated neuronal stimulation in *Drosophila*. We further discuss what is known about DopEcR in other insects and DopEcR orthologs in mammals, implicating their roles in stress responses. Overall, this review highlights the importance of dual GPCRs for catecholamines and steroids in modulating physiology and behavior under stress conditions. Further multidisciplinary studies of *Drosophila* DopEcR will contribute to our basic understanding of the functional roles and underlying mechanisms of this class of GPCRs.

Keywords

stress; GPCR; nongenomic steroid action; catecholamine; *Drosophila*

I. Introduction

The insect dopamine/ecdysteroid receptor, or DopEcR, is a unique G-protein-coupled dual receptor for the catecholamine dopamine and the insect ecdysteroids, ecdysone (E) and 20-hydroxyecdysone (20E) (Srivastava et al., 2005). This receptor mediates unconventional

rapid nongenomic actions of steroids, as well as dopamine signaling, and initiates multiple intracellular signaling pathways including the cyclic adenosine $3',5'$-monophosphate (cAMP), phosphoinositide 3-kinase (PI3K), and mitogen-activated protein kinase (MAPK) pathways, in a ligand-dependent manner. Because DopEcR is primarily expressed in the nervous system (Srivastava et al., 2005; Graveley et al., 2011; Abrieux et al., 2013), and both steroids and catecholamines act as stress hormones in vertebrates (McEwen, 2008), it is intriguing to consider that DopEcR coordinates different signaling pathways to modulate the physiological and behavioral response to stress. This review begins by introducing stress hormones and rapid unconventional steroid responses (section II), and then discusses work on ecdysone and dopaminergic signaling in *Drosophila* stress responses (section III). Following a historical perspective behind the identification and characterization of *Drosophila* DopEcR (section IV), we describe previous studies on DopEcR and provide new evidence showing that DopEcR is required for starvation-induced sleep suppression, neuronal stability during heat stress, and ethanol-induced locomotor behavior (section V). Next, we review the role of DopEcR in other insects (section VI) and its functional orthologs in mammals (section VII). Lastly, we provide perspective on future DopEcR research (section VIII). Together, the experimental findings of DopEcR's function in insects, and comparative mammalian orthologs, provide the foundation for understanding dual GPCRs for catecholamines and steroids in regulation of physiological and behavioral response to stress.

II. Stress hormones and unconventional actions of steroids through G-protein coupled receptors (GPCRs)

The biological term "stress" was first coined more than 80 years ago by the endocrinologist Hans Selye to describe "the non-specific response of the body to any demand for change that potentially disturbs a complex dynamic equilibrium, or homeostasis" (Selye, 1936). Diverse environmental factors and conditions can serve as stressors that trigger a range of physiological and behavioral responses. They include heat, cold, toxic chemicals, starvation, dehydration, sleep deprivation, exposure to predators, social defeat, and many others. Responses to these stressors have evolved to maintain homeostasis and better an organism's chance of survival in hazardous, potentially life-threatening situations. The fundamental molecular and genetic mechanisms underlying stress responses are therefore expected to be conserved among diverse organisms (Singh et al., 2008; Nesse et al., 2016).

Environmental stressors induce changes in, among others, endocrine states and stimulate the secretion of stress hormones such as cortisol. Steroid hormones were originally found to act through transcriptional regulation by nuclear steroid hormone receptors that function as steroid-dependent transcription factors, eliciting biological responses with a time lag of hours or even days (Evans, 1988; Beato, 1989). However, it has been known for a long time that steroids can also act via rapid mechanisms independent of transcriptional regulation, actions referred to as "nongenomic" responses to steroids. For example, it was reported as early as 1939 that estrogen rapidly exerts effects within 3–15 minutes on peripheral blood vessels were reported as early as 1939 (Reynolds and Foster, 1939). Selye (1942) also demonstrated that steroids, including cortisol, have an anesthetic effect in rats within

minutes. Following these early reports, a number of studies have shown that steroid-mediated phenomena can occur very quickly, within seconds to minutes (Blackmore et al., 1990; Winter et al., 1999) and be observed even in cells lacking a functional nucleus, such as erythrocytes, platelets, or spermatozoa (Shivaji and Jagannadham, 1992; Moro et al., 2005; Ivanova et al., 2008).

Steroid hormones use multiple cellular mechanisms to generate rapid nongenomic effects. They can change physiological properties of target cells by influencing membrane fluidity (the viscosity of the lipid bilayer that determines the mobility of lipids and membrane associated proteins) or by directly binding ion channels, including GABA receptors and NMDA receptors (Park-Chung et al., 1997; Whiting et al., 2000; Hosie et al., 2006), to allosterically regulate their function. Under certain conditions, the rapid actions of steroids can be mediated by classical nuclear hormone receptors that localize to the plasma membrane and interact with cytosolic signaling molecules (Prager et al., 2010; Groeneweg et al., 2012). In addition to these mechanisms, it is now accepted that steroids can bind GPCRs and play an important role in nongenomic activation of steroid-triggered signaling pathways. The most well-studied GPCR of this class is the G-protein-coupled estrogen receptor 1 (GPER1) (Prossnitz and Barton, 2011; Feldman and Limbird, 2017). This receptor was initially identified as an orphan GPCR (GPR30) and then renamed GPER1 when it was discovered to mediate nongenomic actions of estrogen (Filardo et al., 2002; Filardo and Thomas, 2005; Revankar et al., 2005; Vivacqua et al., 2006; Revankar et al., 2007; Wang et al., 2008). GPER1 is expressed in many tissues, including reproductive organs, pancreas, liver, heart, arteries, breast, lung, leukocytes, and neural tissue (Hasbi et al., 2005). GPER1 activity affects multiple signaling pathways, including those involving epidermal growth factor receptor (EGFR), extracellular-related kinase (ERK), and inositol-trisphosphate 3-kinase (IP3K), and plays a role in diverse estrogen-dependent processes regulating endocrine, immune, neuronal, and cardiovascular functions. Considering that aldosterone is a more potent agonist of GPER1 than estrogen (Gros et al., 2011), GPER1 may have even wider roles in nongenomic steroid signaling than previously thought. Relevant to this review, GPER1 knockout male mice display reduced anxiety under stressful conditions compared to control males, whereas female mice show altered corticosterone levels and acute stress coping behavior in an estrous cycle-dependent manner (Kastenberger and Schwarzer, 2014). As such, the roles of GPER1 in stress responses are sex- and paradigm-dependent, but the cellular mechanisms by which GPER1 mediates steroid actions in response to stress still remain elusive.

III.   Involvement of ecdysone and dopamine in the stress response in *Drosophila*

Ecdysone.

20-hydroxyecdysone (20E), commonly known as the molting hormone, is the major steroid hormone in insects (Riddiford, 1993). Its secretion from the endocrine ring gland plays a pivotal role in coordinating major developmental transitions, including larval molting and metamorphosis via the 20E-activated nuclear hormone receptor, ecdysone receptor (EcR) (Thummel, 1996; Beckstead et al., 2005; Yamanaka et al., 2013). Although the primary

focus of ecdysone research has been on the regulation of development, accumulating evidence from studies in *Drosophila* suggest that ecdysone signaling has important functions in mature flies as well, particularly in responses to environmental stressors (Ishimoto and Kitamoto, 2011). For example, 20E levels increase in the ovaries and hemolymph of adult females upon exposure to stressors such as starvation, heat, and dehydration (Hirashima et al., 2000a; Rauschenbach et al., 2000; Gruntenko et al., 2005). Activated ecdysone signaling in adult females triggers the arrest of oogenesis (Soller et al., 1999; Terashima and Bownes, 2004), ovulation (Knapp and Sun, 2017), and egg-laying (Gruntenko et al., 2005). Under these unfavorable environments, development-arrested eggs are reabsorbed by the female's body, and the energy and materials derived from the eggs are reutilized for their own survival (Terashima et al., 2005). Stress-induced elevation of 20E is accompanied by decreased ecdysis triggering hormone (ETH) levels, which results in the reduction of octopaminergic input to the reproductive tract and consequently ovulation arrest (Meiselman et al., 2018). In adult males, the social stress of being constantly rejected by sexually unreceptive females increases 20E levels, and this endocrinological change is necessary for the formation of long-term memory represented as courtship suppression (Ishimoto et al., 2009).

20E in adult flies also plays major roles in the regulation of gene expression in response to immune challenge (Flatt et al., 2008). Elevated levels of 20E in response to dehydration leads to adaptive enhancement of the innate immune response, promoting the robust induction of antimicrobial peptide (AMP) genes upon immune challenge (Zheng et al., 2018). Thus, environmental stressors acutely increase 20E levels in adult flies and induce physiological and behavioral transitions that are potentially advantageous for coping with stress. Counterintuitively, flies heterozygous for *EcR* loss-of-function mutations are more resistant to dying from heat, starvation, and oxidative stress, and exhibit a remarkable 50% longer lifespan under controlled laboratory conditions (Simon et al., 2003). Consistent with these findings, when 20E levels are reduced in mutants of an ecdysone biosynthetic gene, a similar lifespan extension is observed and the effects are reversed by feeding of 20E (Simon et al., 2003). These results suggest that ecdysone signaling in wildtype flies may be beneficial in certain contexts but detrimental in others. EcR or ecdysone synthesis mutants with reduced ecdysone signaling may optimize physiological states and health in adult flies to extend lifespan (Simon et al., 2003). However, it is possible that flies with subnormal ecdysone signaling are more vulnerable in the wild where they are more likely to be exposed to various environmental stressors.

Dopamine.

Dopamine is a biogenic catecholamine that functions as a neurotransmitter and hormone in organisms across taxa. Dopamine is produced and released in response to various stressors, which acutely alters animal's physiology and behavior. Dopaminergic signaling in vertebrates governs and modulates a wide range of behavioral processes including motor control, appetite, mood, and motivation (Crocker, 1997; Arias-Carrion et al., 2010; Singh, 2014). Although not the focus of this review, similar roles for dopamine have been extensively studied in flies including locomotion, courtship, sleep/arousal, circadian modulation, temperature preference, drug response, and learning and memory, to name a few

(For reviews please see Kume et al., 2005; Yamamoto and Seto, 2014; Kasture et al., 2018). There are approximately 130 neurons in the adult fly brain that synthesize and release dopamine (Mao and Davis, 2009), and the fly genome encodes four GPCRs serving as dopamine receptors: two D1-like Gs-coupled, *Dop1R1* and *Dop1R2*, one D2-like, *Dop2R*, which is Gi-coupled, and the non-canonical dopamine and ecdysone receptor, *DopEcR* (Gotzes et al., 1994; Feng et al., 1996; Brody and Cravchik, 2000; Srivastava et al., 2005). The canonical receptors have each been implicated in various behavioral responses and are required in temporal-, cell- and pre- and/or post-synaptic manners (Karam et al., 2019).

There is also a profound appreciation for the precise spatiotemporal release of dopamine within the fly central nervous system that underlies behavioral regulation and stress responses (Neckameyer and Weinstein, 2005; Kasture et al., 2018). It was found that dopamine synthesis is altered in response to nutritional, mechanical, and oxidative stress (Neckameyer and Weinstein, 2005). Starvation-induced changes in dopaminergic signaling have further been shown to drive enhanced perception of nutrients (Linford et al., 2015). Dopaminergic signaling also modulates response to high temperatures via FOXO/insulin signaling pathway and juvenile hormone signaling (Hirashima et al., 2000b; Gruntenko et al., 2004; Rauschenbach et al., 2007; Gruntenko et al., 2016). The following sections will elaborate more on the non-canonical dopamine ecdysone receptor, DopEcR.

IV.   Identification and characterization of *Drosophila* DopEcR as a dual GPCR for ecdysteroids and dopamine

Identification.

The *Drosophila melanogaster* gene *CG18314* was originally categorized as an "unclassified biogenic amine receptor" (Brody and Cravchik, 2000) and "nonpeptide orphan receptor" (Hewes and Taghert, 2001). Its product was first singled out as being a prominent target of RNA-editing in the fly transcriptome (Stapleton et al., 2002; Xia et al., 2005), but was soon after recognized for its unusual function as a GPCR that mediates nongenomic actions of ecdysone (Srivastava et al., 2005). Specifically, heterologous expression experiments demonstrated that cell-surface expression of this GPCR leads to cAMP activation, a D1-like response to dopamine, but could additionally elicit rapid ERK signaling in the presence of ecdysteroids. In recognition of its unique dual ligand sensitivity, CG18314 was aptly renamed DopEcR, the dopamine/ecdysteroid receptor (Srivastava et al., 2005). DopEcR contains conserved amino acids in its transmembrane region V that are important for catecholamine binding, and the physiologically relevant concentration of 1 μM of dopamine activated the PI3K/Akt pathway in fly Sf9 and mammalian CHO cells. Competition binding assays were then performed with ecdysteroids, including ponasterone A (PoA), a plant ecdysteroid. Radiolabeled PoA showed saturable binding specificity at $K_D = 10.4 \pm 0.38$ nM, and both ecdysone (E) and 20-hydroxyecdysone (20E) were able to displace PoA. The order of affinity was PoA > E > 20E and neither makisterone A, another insect steroid that activate nuclear ecdysteroid receptors nor dopamine could displace PoA. In addition to ecdysteroid specificity, ecdysteroids dose-dependently inhibited dopaminergic responses (Srivastava et al., 2005).

Gene expression profiling in different tissues and developmental stages revealed that *DopEcR* is highly expressed, particularly within the nervous system, and at the end of embryogenesis, during pupation, and into adulthood (Chintapalli et al., 2007; Ishimoto et al., 2009; Graveley et al., 2011). New evidence also finds *DopEcR* expression in heart tissue (Zheng et al., 2019). Loss-of-function and cDNA expression studies have now confirmed that DopEcR serves many functions in adult behavior, most of which are in response to stress.

Sequence.

The *DopEcR* gene spans a 12,739 bp region on the left arm of the third chromosome in *D. melanogaster*. Three different *DopEcR* transcripts are currently known, which differ in 5' and 3' UTR sequences, but each encodes for the same 322 aa protein sequence. Interestingly, at least 25 different RNA editing sites have been identified between *DopEcR* cDNA and EST sequences (Brody and Cravchik, 2000), but their functional implication remains to be determined. Phylogenetic analysis of the DopEcR protein sequence indicates that it shares greater similarity to vertebrate β-adrenergic receptors than it does to the fly dopaminergic receptor family (Srivastava et al., 2005), suggesting that DopEcR may have unique non-redundant dopaminergic functions compared to the other fly dopamine receptors.

Ligands.

As mentioned in the next section, DopEcR is necessary for sensing the starvation-induced dopamine released onto primary sugar-sensing neurons (Inagaki et al., 2012). It is also likely that DopEcR mediates dopaminergic signaling in starvation-induced sleep suppression (Fig. 1) and alcohol-induced hyperactivity (Fig. 3). These findings suggest that dopaminergic activation of DopEcR ultimately enhances neuronal excitability and potentiation. They also suggest that DopEcR is poised to locally respond within primary and higher order neuronal circuits to produce generalized or specific behavioral outcomes.

In addition to dopamine, the other ligand of DopEcR, is the insect hormone ecdysone. It is generally believed that lipophilic steroid hormones, such as 20E, can enter cells through the plasma membrane by simple diffusion. However, a recent study demonstrated that Ecdysone Importer (EcI), an evolutionarily conserved solute carrier organic anion transporter, is required for 20E transport across the plasma membrane (Okamoto et al., 2018) to bind EcRs in the cytoplasm. DopEcR is currently the only non-canonical steroid GPCR identified in flies, and its activation by ecdysteroids on the plasma membrane elicits rapid (within minutes) nongenomic intracellular signaling events independent of canonical EcR (Srivastava et al., 2005; Evans et al., 2014). This function has been shown to mediate ecdysteroid responses *in vivo* including experience-dependent courtship suppression (Ishimoto et al., 2013) and alcohol-induced sedation (Petruccelli et al., 2016). DopEcR has also recently been implicated in mediating aldosterone steroid responses in adult nephrocytes, which filter hemolymph and have been shown to share functional, morphological, and molecular features with vertebrate podocytes (Zheng et al., 2019). Since *DopEcR* expression is detected, albeit at lower levels, in non-neural tissues such as the midgut, fat body, salivary gland, and testis (Leader et al., 2018), it is possible that DopEcR

plays novel roles in these tissues in a dopamine- and/or ecdysone-dependent manner. Loss of DopEcR function does not appear to grossly impact *D. melanogaster*'s developmental processes despite detectable mRNA expression of *DopEcR* during development (Petruccelli et al., 2016). A recent paper, though, has thoroughly implicated DopEcR in the development of the lepidopteran agricultural pest *Helicoverpa armigera* (Kang et al., 2019)(see 'Roles of DopEcR in other insects'). It is worth reexamining a potential role of DopEcR in *Drosophila* development particularly under unfavorable conditions that may trigger stress responses.

Intracellular signaling.

Whether activated by dopamine, ecdysone or independent of a ligand, DopEcR can trigger rapid intracellular signaling events. Both PI3K/Akt and MAPK/ERK signaling was initiated in response to dopamine and ecdysone, respectively, in *in vitro* heterologous expression systems (Srivastava et al., 2005). *In vivo*, cAMP and ERK signaling have been functionally observed to be downstream of DopEcR (Ishimoto et al., 2009; Petruccelli et al., 2016). *In vivo*, bioluminescent calcium imaging revealed elevated calcium responses to nicotinic stimulation within mushroom body (MB) neurons lacking DopEcR, indicating that DopEcR signaling normally functions to negatively modulate MB excitability (Lark et al., 2017). Furthermore, dopamine signaling likely drives these inhibitory changes in the medial lobes of the MBs, whereas ecdysone enhances calcium signaling primarily in the calyx and cell body region of the MBs. Together these findings suggest that DopEcR mediates complex changes to MB excitability in an agonist-specific manner. In general, GPCRs are known to extensively interact with ion channels to influence neuronal excitability. Interactions include receptor-channel co-trafficking, channel modulation by G-α or -β/γ molecules, post-translational modifications, and changes in lipid membrane dynamics (Inanobe and Kurachi, 2014). Although the particular G-proteins coupled to DopEcR currently remain unknown, the evidence for DopEcR-ion channel interactions or crosstalk is mounting (e.g., heat-induced seizure and defective habituation in *DopEcR* loss-of-function mutants). Revealing these possible interactions will help elucidate DopEcR's influence in excitable cells.

V. Functions of *Drosophila* DopEcR in stress responses

In *Drosophila*, *DopEcR* is dispensable for survival, at least under controlled laboratory conditions (Petruccelli et al., 2016). Loss-of-function mutants of *Drosophila DopEcR* exhibit no gross developmental or anatomical defects. Nonetheless, their physiological and behavioral responses to some environmental stressors are considerably different from those of genetic controls, indicating the critical function of DopEcR in various stress responses.

Starvation.

DopEcR's first documented function in behavior was for the dopaminergic modulation of enhanced proboscis extension response (PER) following starvation (Inagaki et al., 2012). The PER assay has been used to evaluate taste sensitivity as well as the overall motivation to consume food in flies (Dethier, 1976). Inagaki and colleagues show that starvation increases dopaminergic release onto Gr5a sugar-sensing neurons, and that DopEcR is required in these sensory neurons to mediate evoked calcium in response to extra dopamine release and ultimately increase PER (Inagaki et al., 2012). Thus, DopEcR is required for starvation-

dependent enhancement in sugar sensitivity and PER behavioral response. Building upon this finding, a follow up study by Inagaki and colleagues showed that DopEcR is not required for the reciprocal starvation-induced reductions in bitter compound sensitivity, which is instead mediated by sNPF (short neuropeptide F) via sNPFR (Inagaki et al., 2014). DopEcR is also required for increasing appetite in flies chronically fed sucralose. Sucralose is a non-nutritive synthetic sweetener, which is known to trigger a neuronal fasting response in control flies and increases the motivation to eat (Wang et al., 2016). Thus, DopEcR appears to be necessary in specific pathways in the nervous system for higher-order detection and/or response to starvation.

Since starvation is potentially life-threatening, many organisms under starvation conditions will forgo sleep in order to obtain food for survival. This behavior, known as starvation-induced sleep suppression (SISS), has been observed across phyla. In *Drosophila*, a mild starvation, or fasting state, promotes wakefulness by largely impairing sleep initiation and prolonging wake-bout lengths (Keene et al., 2010; McDonald and Keene, 2010; Melnattur and Shaw, 2019; Yurgel et al., 2019). SISS is most readily observed in the daytime hours just after flies are transferred to wet starvation conditions. Consistent with the critical role of DopEcR in enhancing feeding behavior under starvation, we found that *DopEcR* loss-of-function mutants were defective in SISS behavior. As shown in Fig. 1A and 1B, *DopEcR* hypomorphic (*DopEcR^{PB1}*) and null (*DopEcR^{Gal4}*) mutants showed less sleep suppression than control flies. It is also important to note that baseline sleep patterns also appear to be structurally different, with mutants showing reduced sleep in the middle of the day and less overall evening sleep. Quantification of the total daytime sleep was consistent across control and *DopEcR* flies on the baseline day, but only controls showed a statistically significant loss of sleep upon starvation (Fig. 1B). The percent change response from baseline confirmed that *DopEcR* mutants displayed statistically significant differences from control flies (Fig. 1C). This suggests that DopEcR is necessary for wake-promoting behavior in response to starvation. Since dopamine is known to promote wakefulness in *Drosophila* under regular fed conditions (Andretic et al., 2005; Kume et al., 2005), DopEcR likely mediates SISS through a dopaminergic signal. Elimination of dopamine via application of dopamine synthesis inhibitor 3-iodotyrosine (3IY) was shown to suppress the starvation-induced lengthening of wake-bouts in control flies (Fig. 1D), indicating the critical involvement of dopamine signaling in SISS, possibly via DopEcR. Mutants for the other conventional dopamine receptors *Dop1R1*, *Dop1R2*, and *Dop2R*, displayed normal, or even exaggerated, SISS behavior (Fig. 1C). Thus, DopEcR serves a unique wake-promoting role in response to starvation. Ecdysone signaling can also influence sleep via EcRs (Ishimoto and Kitamoto, 2010), but whether DopEcR mediates an ecdysteroid signal to control SISS remains to be investigated.

Heat.

Heat is another commonly experienced environmental stressor. Insects are ectotherms ("cold-blooded" organisms), and thus internal body temperatures are especially vulnerable to change in the ambient temperature. Since temperature has a significant impact on conductance and gating kinetics of ion channels (Kiernan et al., 2001; Buzatu, 2009; Chowdhury et al., 2014), heat treatment may acutely induce abnormalities in neural activity

and behavior. Mutations in ion channel genes often increase the sensitivity to heat and result in heat-induced behavioral phenotypes such as paralysis and seizure. $para^{ts1}$, a recessive loss-of-function, temperature-sensitive mutation in the fly voltage-gated sodium channel gene *paralytic* (*para*), causes an immediate, but reversible, paralysis of adult flies when they are shifted from 22°C to 29°C (Suzuki et al., 1971). $para^{GEFS+}$ is another *para* allele which harbors a gain-of-function mutation that mimics the human mutation underlying an epilepsy syndrome called generalized epilepsy with febrile seizures plus (GEFS+). Upon exposure to 40°C homozygous or hemizygous $para^{GEFS+}$ flies display seizure-like behavior, including leg twitches, failure to maintain standing posture, wing buzzing, and occasional abdomen curling (Sun et al., 2012). Remarkably, we found that *DopEcR* mutants show behavioral changes reminiscent of $para^{GEFS+}$ responses upon heat stress (Fig. 2A). Mutants displayed robust heat-induced seizure-like behavior with ~90% of homozygous $DopEcR^{Gal4}$ null mutants and ~40% of homozygous $DopEcR^{PB1}$ hypomorphic mutants showing seizure-like activity within two minutes of 40°C heat treatment. After the onset of heat, $DopEcR^{Gal4}$ flies rapidly lost postural control and fell over onto their backs. They often extended or buzzed their wings and repeatedly kicked their legs or curled their abdomens up into the air. *DopEcR* trans-heterozygotes ($DopEcR^{Gal4}$/$DopEcR^{PB1}$) expectantly failed to compliment, and DopEcR appeared to be haplosufficient for this trait since heterozygous mutants ($DopEcR^{Gal4}$/+ and $DopEcR^{PB1}$/+) showed little seizure-like behavior upon heat treatment (Fig. 2B). Although DopEcR is required to maintain neuronal homeostasis under extreme heat, *DopEcR* mutants have normal heat place memory (Ostrowski et al., 2015), suggesting that they have intact noxious heat sensation.

Alcohol.

Alcohol is a natural by-product of fermentation, and many organisms are sensitive to its disinhibiting effects. Flies are ecologically drawn to low-levels of alcohol since it represents good food sources and egg-laying locations. Similar to mammals, as internal levels of ethanol rise, flies display characteristic loss-of-postural control and sedation behavior (Wolf et al., 2002). DopEcR has previously been found to mediate an ecdysteroid response in the context of acute ethanol exposure (Petruccelli et al., 2016). During 50% ethanol vapor exposure, DopEcR mutants lost postural control around the same time as control flies, but sustained shaking and re-righting movements long after control flies sedated. DopEcR was required in cholinergic or peptidergic neurons, but not MB neurons, to promote ethanol-induced sedation, and 20E feeding negatively modulated this function (Petruccelli et al., 2016; Aranda et al., 2017). Beyond its role in ethanol-induced sedation, we show here that DopEcR is also required for ethanol-induced locomotion (Fig. 3A). Loss-of-function *DopEcR* mutants displayed enhanced and prolonged hyperactivity during 50% ethanol vapor exposure compared to control flies. This phenotype is interestingly opposite of the previously published phenotype of a *Dop1R1* hypomorphic allele, $Dop1R1^{PL00420}$ (Kong et al., 2010), suggesting that DopEcR counteracts Dop1R1 signaling. To test the genetic interaction between these receptors, double mutant $Dop1R1^{PL00420}$ $DopEcR^{Gal4}$ flies were created and assayed in 73% ethanol vapor. Surprisingly, double mutants showed a dramatically exaggerated hyperlocomotor response to ethanol (Fig. 3B), suggesting a complex neuronal hierarchy or cellular interaction between DopEcR and Dop1R1. Lastly, to determine if DopEcR is indeed mediating a dopaminergic signaling in this behavior, we

assessed the response of dopamine transporter *fumin* (*DAT^{fmn}*) mutants (Kume et al., 2005) and *DAT^{fmn}*; *DopEcR^{Gal4}* double mutants. Double mutants showed a similar response to that of *DopEcR^{Gal4}* mutants (Fig. 3C) supporting the model where DopEcR acts downstream of synaptic dopamine. It is also important to note that baseline locomotion during air exposure may, itself, contribute to subsequent ethanol-induced responses, but to our knowledge this correlation has not yet been extensively investigated. Future studies are needed to elucidate the molecular and cellular underpinnings of interactions between DopEcR and Dop1R1, and whether DopEcR mediates ecdysteroid signaling to influence ethanol-induced locomotion.

Courtship rejection.

Drosophila males are innately able to display a stereotypical sequence of courtship rituals toward females (Hall, 1994). However, male courtship behavior is also subject to modification through social experience with females. Recently mated females are sexually unreceptive and resist male courtship advances by displaying a series of characteristic rejection behaviors, including extruding their ovipositor, kicking, or decamping (Villella and Hall, 2008). Receiving rejections from unreceptive females is considered a form of social stress for the courting male, and repeated courtship rejection ultimately suppresses male's courting behavior, a behavior exploited for a learning and memory assay in *Drosophila* (Siegel and Hall, 1979). The first evidence of DopEcR mediating a nongenomic ecdysteroid response *in vivo* was for its role in experience-dependent courtship suppression (Ishimoto et al., 2013). Specifically, DopEcR in the MB neurons is required for 20E-induced changes in cAMP that necessitates short-term courtship memory formation and/or expression. Later, calcium imaging experiments demonstrated that ecdysone enhances nicotine-induced Ca^{2+} signaling in the MB calyces and somas in a DopEcR-dependent manner, supporting the possibility that DopEcR plays an important role in experience-dependent modification of the physiological properties of MB neurons (Lark et al., 2017).

Repetitive neuronal stimulations.

In adult flies, visual or mechanical stimulation activates the descending giant fiber (GF) neurons and triggers the stereotypical jump-and-flight escape response (Tanouye and Wyman, 1980; Wyman et al., 1984). Direct electrical stimulation of the brain can bypass sensory receptors and activate the GF pathways to set off the escape response. Strong electrical stimulation can directly trigger GF activation (short-latency response), whereas lower intensity stimulation indirectly activates the GF pathway through GF afferents in the brain (long-latency response) (Elkins and Ganetzky, 1990). The lowest stimulation intensities required for triggering short- and long-latency responses are not different between *DopEcR* loss-of-function mutants and control flies, indicating that reduced DopEcR does not significantly affect the overall neuronal sensitivity of the GF pathway. However, when the GF pathway is repetitively stimulated, *DopEcR* mutants show functional abnormalities; the minimum time required for the GF system to recover from the 1st stimulus and fire a response to the 2nd stimulus (the refractory period) is significantly reduced in *DopEcR* mutants compared to control flies (Ishimoto et al., 2013). This finding implies that neuronal circuits in *DopEcR* mutants are less vulnerable to activity-dependent modifications than the relevant circuits in control flies. With repeated delivery of electrical stimulation across the brain, the GF pathway undergoes habituation and the probability of a motor output

significantly decreases (Engel and Wu, 1996). Analysis of habituation clearly demonstrated defects of the *DopEcR* mutant circuits in responses to repetitive neuronal stimulations. In contrast to control flies that became rapidly habituated to 5-Hz stimulation of the brain and failed to respond to the brain stimulation, *DopEcR* mutants consistently showed a significant delay in habituation (Ishimoto et al., 2013). These electrophysiological abnormalities in *DopEcR* mutants may underlie their defects in courtship memory and other behaviors.

VI.   Roles of DopEcR in other insects

The diverse roles of DopEcR homologs have been implicated in insects other than *Drosophila*. These include biological processes such as phase trait polyphenism, reproduction, apoptosis, cAMP metabolism, and ecdysis-associated behavioral quiescence, some of which are related to stress response (Elmogy et al., 2004; Elmogy et al., 2006; Chen et al., 2010; Abrieux et al., 2013; Kang et al., 2019).

Gregarious transformation in Locusta migratoria.

Phase polyphenism is a phenomenon where animals of a single species undergo great phenotypic changes in response to environmental cues (Applebaum and Heifetz, 1999). In the case of the migratory locust *Locusta migratoria*, increased population density drives a transformation from the solitarious to gregarious phase within a generation with marked metabolic and neurobiological changes, resulting in their dark coloration, swarming, and dramatic mass migrations which can lead to terrible agricultural losses (Gray et al., 2009). This transformation involves changes in both transcription of catecholamine-related genes (Ma et al., 2011) and ecdysteroids titer (Tawfik and Sehnal, 2003). *DopEcR* was upregulated in gregarious-phase locusts compared to solitarious-phase locusts (Chen et al., 2010). While the exact role of *DopEcR* in the phase transformation is unclear, its upregulation in the gregarious phase together with changes in ecdysteroid levels indicates that ecdysone signaling through DopEcR may play a role in helping locusts properly manage their phenotypic responses to a high-density environment, which is considered socially stressful for locust (Roessingh et al., 1998; Simpson et al., 2001).

Age-dependent courtship plasticity in Agrotis ipsilon.

As previously mentioned DopEcR is required for experience-dependent courtship suppression in *D. melanogaster* males. A similar situation is found in the moth *Agrotis ipsilon*, where DopEcR was shown to modulate age-dependent behavioral plasticity (Abrieux et al., 2013). *A. ipsilon* males eclose from pupal cases sexually immature and over the course of several days, gain competence to be attracted to female-derived sex pheromones. This age-dependent plasticity is partially dependent upon ecdysteroids and dopaminergic signaling through *DopEcR*, as injection of *DopEcR* dsRNA in male moths inhibited behavioral attraction to female-derived sex pheromones (Abrieux et al., 2013). Sex pheromones activate neurons in the male antennal lobes in an age-dependent fashion and concentrations of the sex pheromone necessary to evoke neuronal responses decrease with age (Anton and Gadenne, 1999). Behavioral response to sex pheromone is augmented by treating male moths with either dopamine or 20E, and this response requires DopEcR

expression (Abrieux et al., 2013). Together these findings highlight the role of DopEcR-mediated dopamine and ecdysone actions in the plasticity of sexual behavior in *A. ipsilon*.

Larval feeding and pupation in Helicoverpa armigera.

In the agricultural pest *Helicoverpa armigera*, DopEcR has been shown to function as a switch of sorts that integrates dopamine and 20E signals to decide whether a larva will continue to actively feed and grow or enter behavioral quiescence and begin pupation (Kang et al., 2019). This is accomplished through the antagonistic effects of 20E upon DopEcR-dependent dopamine signals. The 20E titer is low during larval feeding and high during wandering stages, while the dopamine titer is opposite to it; high during larval feeding and low during wandering stages. When dopamine is the dominant DopEcR ligand during larval feeding, protein kinase B (Akt) is phosphorylated and activated, thus promoting cell-cycle progression, cell proliferation, and the continuation of larval development. In the hours preceding pupation, hemolymph dopamine titer decreases while 20E titer increases, thus biasing the action of DopEcR towards 20E-dependent effects and life-stage progression. Increased levels of 20E antagonizes phosphorylation of Akt and instead promotes 20E-responsive gene transcription indirectly by promoting phosphorylation of USP1, which enhances EcR-B1/USP1 binding to ecdysone response elements. 20E signals lead to behavioral quiescence, apoptosis, and eventual metamorphosis. This study by Kang et al. (2019) revealed that *DopEcR* is necessary for coordinating cellular and behavioral events to complete pupation in *Helicoverpa armigera* and demonstrated, for the first time, the functional interaction between genomic and nongenomic ecdysone signaling pathways.

Ecdysteroid-induced rapid increase in cAMP levels in Bombyx mori.

In the silkworm *Bombyx mori*, a population of membrane ecdysteroid receptors was identified within cells of the anterior silk glands (ASGs) using tritiated steroid ligands (Elmogy et al., 2004). At pupariation the silkworm uses ASGs to spin their cocoons, but shortly after pupariation the ASGs undergo programmed cell death in response to high ecdysteroid levels. Initially, these ecdysteroids act genomically to begin the sequence of ASG apoptosis, but the sequence also requires nongenomic action of steroids to complete the lysis (Terashima et al., 2000). Ecdysteroid treatment of ASGs triggers an increase of intracellular cAMP, a critical second messenger, that is not abolished by inhibition of transcription, indicating that ASG cAMP induction is likely due to the nongenomic effects. Whether this cellular response requires *DopEcR* to mediate these effects has yet to be determined (Elmogy et al., 2006). Similar increases in cAMP in response to ecdysteroids have been observed in other insects (Applebaum and Gilbert, 1972; Sass et al., 1983), and the rapid nature of cAMP induction makes it an ideal mechanism to mediate an animal's responses to a dynamic stressful environment.

VII. Functional counterparts and potential orthologs of DopEcR in vertebrates.

Although there is little sequence similarity between insect DopEcR and the aforementioned mammalian GPER1, these two GPCRs are considered functional counterparts because they share several key characteristics in terms of their intracellular localization, ligands,

secondary signaling properties and pharmacology (Srivastava et al., 2005; Evans et al., 2014). When expressed in heterologous cell lines, both DopEcR and GPER1 are localized to the plasma membrane but are rapidly internalized into endosomal compartments. Similar to DopEcR, GPER1 is also activated by steroids (i.e., 17β-estradiol, and aldosterone) and dopamine. These ligands can differentially trigger distinct downstream cascades through both receptors, thus providing unique molecular hubs for steroid and dopamine actions.

Sequence analysis with the DIOPT (*Drosophila* RNAi Screening Center Integrative Ortholog Prediction Tool, http://www.flyrnai.org/diopt) predicts two putative mammalian orthologs of DopEcR, GPR52 (amino acid sequence identity: 26%, similarity: 42%) and GPR21 (identity: 24%, similarity: 42%). Both are orphan GPCRs – receptors whose endogenous ligands have yet to be identified. A study showed that reduced GPR52 function suppresses Huntington's disease (HD) phenotypes in both patient iPS-derived neurons and in an *in vivo* *Drosophila* HD model (Yao et al., 2015). In the latter study, DopEcR was referred to as "*Drosophila* GPR52" and examined as the functional equivalent of mammalian GPR52. More recent experiments found that expression levels of mutant huntingtin (HTT) were reduced and HD-associated behavioral phenotypes were mitigated in a mouse model of HD when an additional GPR52 mutation was introduced (Huang and Gitler, 2018; Song et al., 2018). Since chronic stress has been implicated in the onset and progression of HD (Mo et al., 2014), DopEcR's correlation to stress-induced responses and the disease severity is intriguing to consider.

GPR21, another potential mammalian ortholog of DopEcR, is preferentially expressed in neurons and responds to plasmalogens (Pls), lipid ligands, albeit not steroids (Hossain et al., 2016). PIs are the special glycerophospholipids that are enriched in the brain and can protect neuronal cells against the damaging effects of stress (e.g., oxidative stress) by enhancing phosphorylation of Akt and ERK (Nagan and Zoeller, 2001). The Pls-mediated phosphorylation of ERK was inhibited in GPR21 knockdown cells, and conversely, GPR21 overexpression enhanced Pls-mediated phosphorylation of ERK and Akt. These results indicate that GPR21 is involved in stress response through modulating phosphorylation (Hossain et al., 2016). To the best of our knowledge, the response of DopEcR to PIs has not yet been investigated.

VIII. Future perspectives

A number of interesting questions regarding DopEcR's function remain to be answered. First, the intracellular distribution of DopEcR in various cell types, particularly in neurons, is still unclear. DopEcR is expected to be present on the plasma membrane, but whether the receptor is enriched around the cell soma, lining axons, or found in dendrites has yet to be addressed. Furthermore, whether DopEcR acts pre- or post-synaptically to modulate specific behaviors will also need to be determined.

Another unanswered question is how DopEcR is activated by endogenous ligands, particularly ecdysteroids. Adult *Drosophila* can synthesize ecdysteroids within the female ovary (Gaziova et al., 2004, Domanitskaya et al., 2014) and from Malpighian tubules in response to desiccation stress (Zheng et al., 2018), but other adult tissues that produce and

release ecdysteroids requires further investigation. The developmental release of ecdysone from the steroidogenic prothoracic gland is similar to that of neurotransmitters from neurons, mediated by calcium-regulated vesicular trafficking (Yamanaka et al., 2015). This raises the interesting possibility that ecdysteroids are synthesized and stored in neurosecretory cells, and released as neurosteroids in an activity-dependent manner.

Equally important is the question of how DopEcR-mediated dopamine and ecdysone signaling interact under various stress conditions. While Srivastava et al. (2005) showed that ecdysone inhibited dopamine-associated downstream signaling in culture, this characteristic bias toward ecdysone may differ *in vivo* and even vary by tissues and brain regions. Additionally, a growing body of work has shown that allosteric modulation of GPCRs may confer bias on or differentially modulate multiple downstream signaling pathways (Foster and Conn, 2017). In the context of DopEcR, this may suggest that the downstream signaling associated with dopamine and ecdysone is regulated dynamically and flexibly. Equally intriguing still, as suggested by the work of Kang et al. (2019) in *Helicoverpa armigera*, the dual ligand capacity of DopEcR may serve as a sort of switch or coincidence detector alone or in league with other dopamine receptors to initiate selective responses to stressors. Clearly there remains much to explore in regard to the specificity and spatiotemporal nature of DopEcR's signaling mechanisms.

An additional question left to be addressed concerns the possible role of DopEcR in *Drosophila* development. Ecdysis (molting) is arguably a stressful event. Many arthropods become behaviorally quiescent before a molt, suggesting that ecdysteroid changes muscle excitability. In support of this, ecdysteroid application to the larval neuromuscular junction (NMJ) acutely reduced the size of postsynaptic currents by reducing the number of synaptic vesicles released by motor neurons, but not changing quantal content (Cooper and Ruffner, 1998; Ruffner et al., 1999). To the best of our knowledge, the role of DopEcR in this context has not yet been investigated.

The findings presented here provide a novel framework for dissecting GPCR-mediated steroid signaling at the molecular and cellular levels. Furthermore, future analysis of the functional interplay between genomic and nongenomic steroid signaling pathways is expected to reveal novel mechanisms through which steroid hormones regulate responses to stressors and other biological phenomena.

Acknowledgements

Abbreviations

Akt	protein kinase B

AMP	antimicrobial peptide
ASG	anterior silk gland
cAMP	cyclic adenosine monophosphate
DopEcR	dopamine/ecdysteroid receptor
E	ecdysone
20E	20-hydroxyecdysone
EcI	Ecdysone Importer
EcR	ecdysone receptor
EGFR	epidermal growth factor receptor
ERK	extracellular-related kinase
ETH	ecdysis triggering hormone
FOXO	forkhead box subfamily O transcription factors (FOXO)
fmn	fumin
GF	giant fiber
GPER1	G-protein-coupled estrogen receptor 1
GABA	γ-aminobutyric acid
GPCR	G-protein coupled receptor
HD	Huntington's disease
HTT	Huntingtin
IP3K	inositol-trisphosphate 3-kinase
3IY	3-Iodotyrosine
MB	mushroom body
NMDA	N-methyl-D-aspartate receptor
PoA	ponesterone A
SISS	starvation-induced sleep suppression
USP	Ultraspiracle

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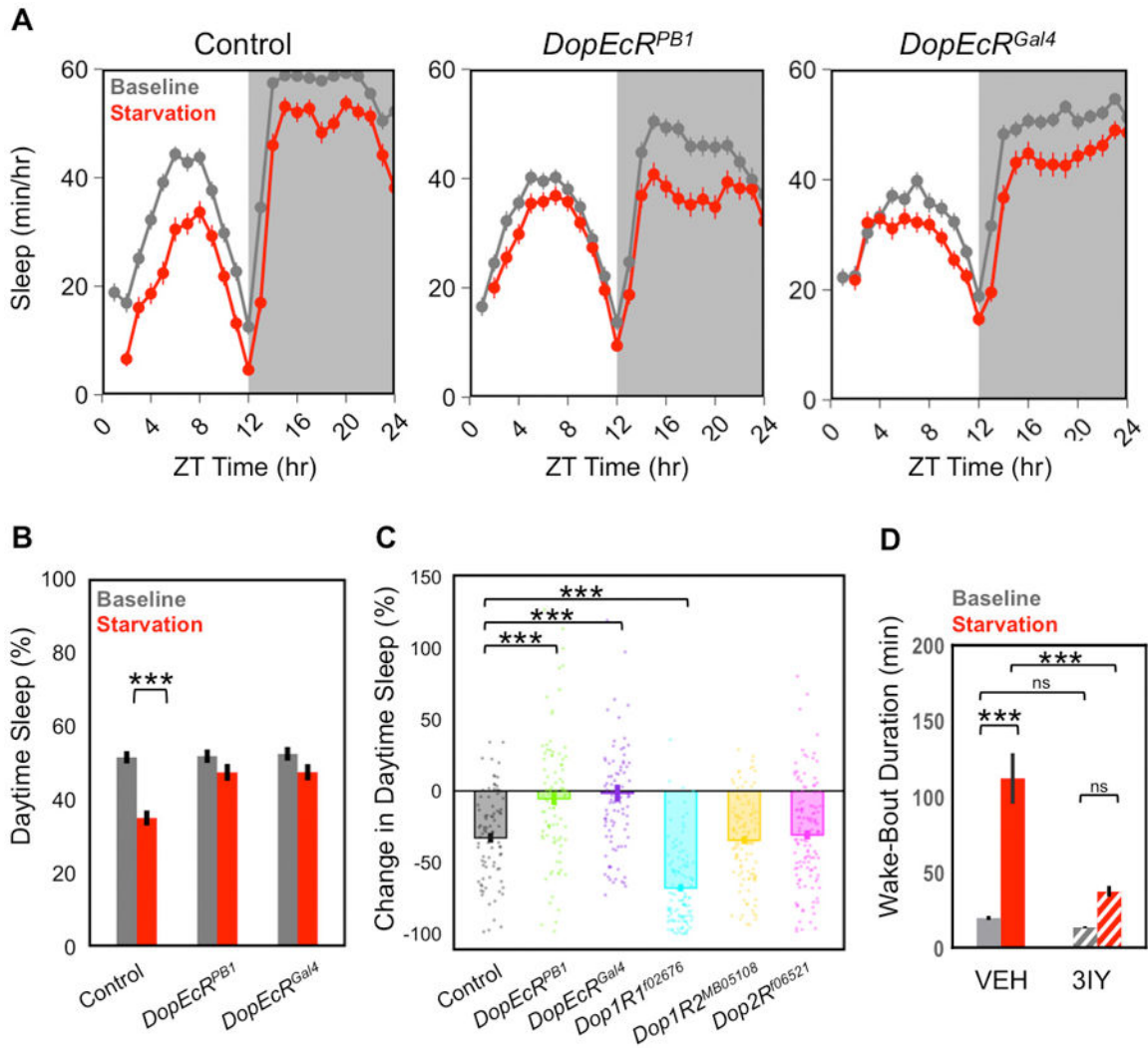
Figure 1: *DopEcR* is required for starvation-induced sleep suppression.
A) Sleep profiles of homozygous *DopEcR* hypomorphic mutants (*DopEcR^{PB1}*, n = 95), null mutants (*DopEcR^{Gal4}*, n = 90), and control flies (*w^{1118}*, n = 88) during a baseline (fed) day and the following starvation days. **B)** The amount of daytime (1–12 ZT hr) sleep in (A) on baseline and starvation days showed a statistically significant SISS response in *w^{1118}* controls, but not in *DopEcR^{PB1}* and *DopEcR^{Gal4}* mutants. **C)** The percent change in sleep was determined for *w^{1118}*, *DopEcR^{PB1}*, *DopEcR^{Gal4}*, *Dop1R1^{PL00420}* (n = 115), *Dop1R2^{MB05108}* (n = 116), and *Dop2R^{f06521}* (n = 126) flies. *DopEcR* mutants lacked SISS behavior, *Dop1R1* mutants showed enhanced SISS, and *Dop1R2* and *Dop2R* mutants showed normal SISS as compared to controls. **D)** Vehicle-treated flies (*w^{1118}*, n = 82) showed a significant increase in wake-bout duration in response to starvation, whereas 6 mM 3IY treated flies (*w^{1118}*, n = 105) did not display this extension in response to starvation.
**Experimental Methods:** Flies were maintained in standardized conditions (25°C, 65% humidity, 12 hr day/night cycle) and aged to 3–5 days old. Both *DopEcR* mutants were backcrossed to *w^{1118}*. Mated female flies were gently mouth pipetted into Drosophila activity monitor (DAM) glass vials (5 × 65 mm) containing 5% sugar 1% agar on a baseline

day and then flies were manually flipped to 1% agar for a starvation day at Zeitgeber Time 0 (ZT 0 hr). For pharmacological manipulations, 3IY (Sigma-Aldrich, St. Louis, MO) or vehicle (1N HCl) was fed via addition to standard food for two days prior to experiment and throughout the experiment. Activity counts per minute were recorded and sleep was defined as 5 minutes or more of inactivity (Hendricks et al., 2000; Shaw et al., 2000). Roughly 2–3 experiments were performed for each genotype; averages and SEMs are plotted; ANOVA with Tukey posthoc tests were performed in R. A two-way ANOVA with tukey's multiple comparison test was performed in prism for the pharmacological assay.

p<0.01,*p<0.001. *DopEcR^{PB1}* (BDSC #10847); *DopEcR^{Gal4}* (Petruccelli et al., 2016); *w^{1118}* (VDRC #6000); *Dop1R1^{PL00420}* (BDSC #19491); *Dop1R2^{MB05108}* (BDSC #24743); *Dop2R^{f06521}* (Exelixis f06521).
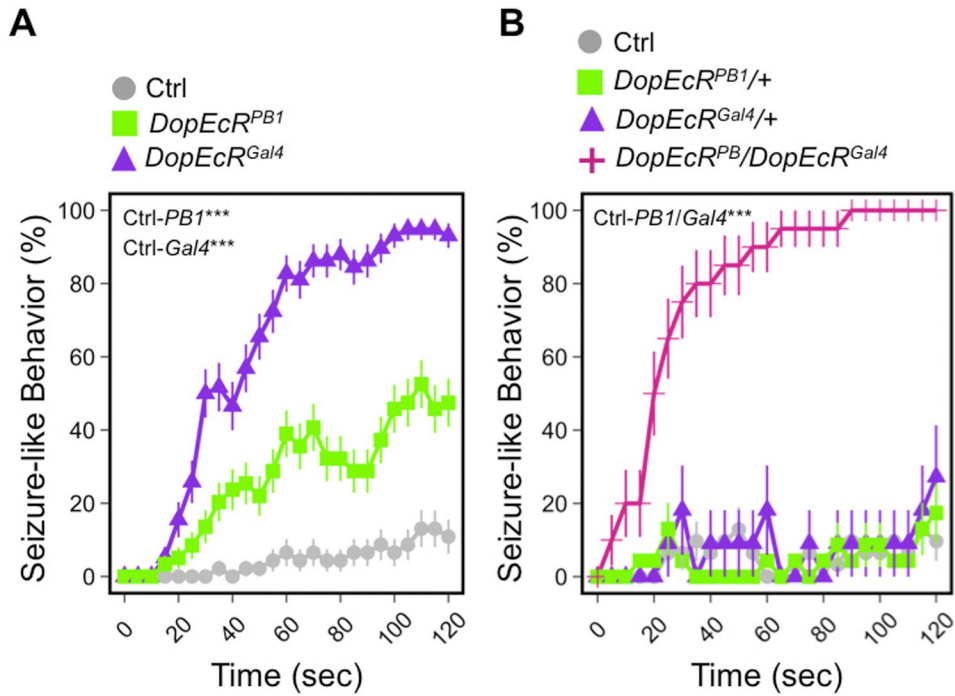
Figure 2: Loss-of-function *DopEcR* mutants exhibit a heat-induced seizure-like phenotype.
A) Homozygous hypomorphic *DopEcR^PB1* (n = 59) and null *DopEcR^Gal4* (n = 46) mutants were more susceptible than w^{1118} (n = 58) control flies to heat-induced seizure behavior. **B)** Heterozygous *DopEcR^PB1* /+ (n = 33) and *DopEcR^Gal4* / + (n = 20) flies behaved similarly to control w^{1118} / + (n = 35), suggesting haplosufficency of *DopEcR*, whereas trans-allelic heterozygotes *DopEcR^PB1* / *DopEcR^Gal4* (n = 45) expectantly failed to compliment and showed heat-induced seizure susceptibility. **Experimental Methods:** Fly cultures were maintained in standardized conditions (25°C, 65% humidity, 12 hr day/night cycle) and aged to 5-days-old. Flies were then placed into small glass vials (15 mm x 45 mm), allowed to acclimate for 5–15 minutes, and then submerged in a 40°C water bath for 2 minutes. Behavior was scored at 5-second intervals. Flies were scored as having a 'seizure' if they had lost posture and fallen over, or exhibited abdomen curling and wing buzzing behaviors. 2–3 experiments were performed for each genotype and male and female data were combined for genotypic averages; averages and SEMs are plotted; ANOVA repeated measures tests with Tukey posthoc tests were performed in R. ***p<0.001.
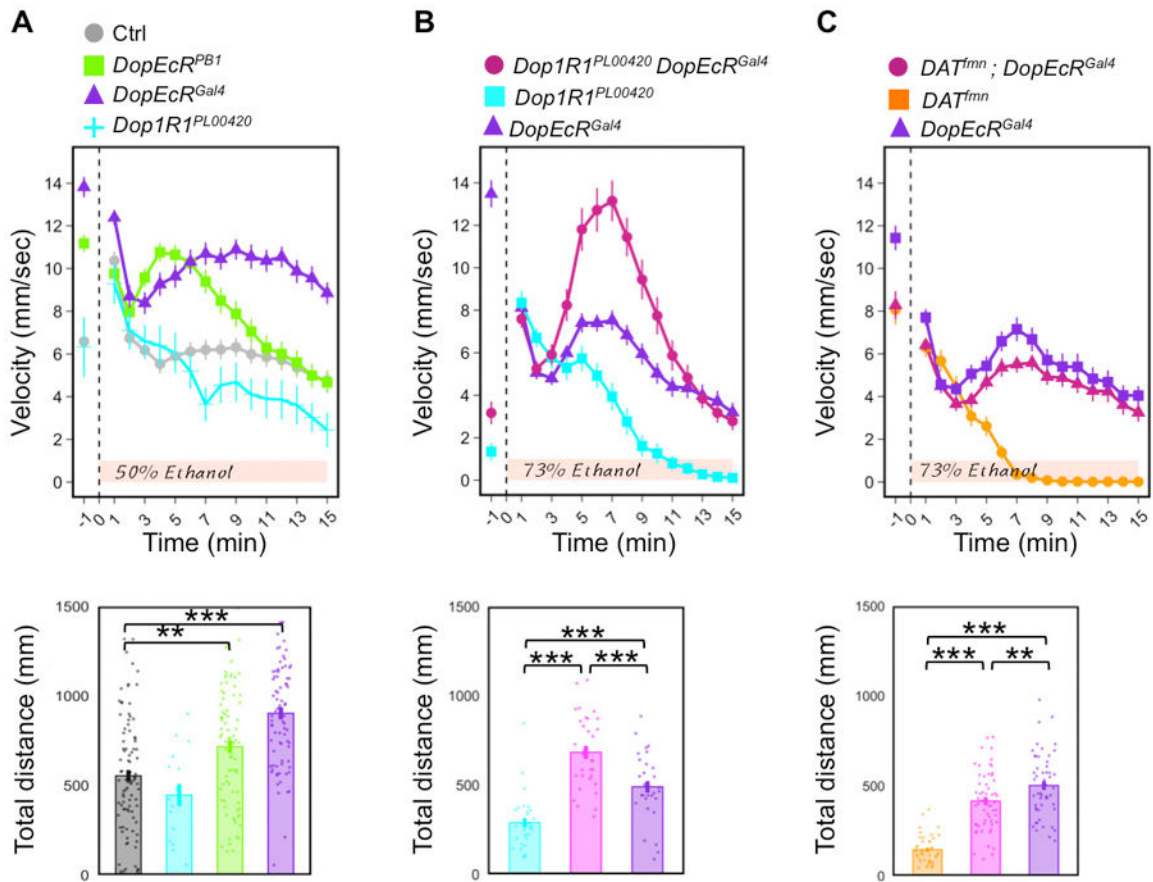
Figure 3. DopEcR mutants display abnormal ethanol-induced locomotion.
A) Homozygous hypomorphic $DopEcR^{PB1}$ (n = 80) and null $DopEcR^{Gal4}$ (n = 80) mutants showed greater locomotor activity than control w^{1118} (n = 95) flies in response to 50% ethanol vapor, whereas $Dop1R1^{PL00420}$ (n = 16) mutants displayer hypo-locomotor responses. **B)** Double mutant $Dop1R1^{PL00420} DopEcR^{Gal4}$ (n = 32) displayed a robustly higher locomotor activity than either $DopEcR^{Gal4}$ (n = 32) and $Dop1R1^{PL00420}$ (n = 31) single mutants flies upon exposure to 73% ethanol vapor. **C)** Double mutant DAT^{fmn} ; $DopEcR^{Gal4}$ (n = 56) displayed a robustly higher locomotor activity than either DAT^{fmn} (n = 44) and $Dop1R1^{PL00420}$ (n = 52) single mutants flies upon exposure to 73% ethanol vapor.
Experimental Methods: Fly cultures were maintained in standardized conditions (25°C, 65% humidity, 12hr day/night cycle) and aged to 3–5-days-old. Male flies were loaded in DAM glass vials, placed in a custom-made plexiglass chamber and delivered 1 minute of humidified air and then 15 minutes of ethanol vapor. Videos were recorded using a LogiTech webcam and analyzed with pySolo tracking (Gilestro, 2012). 2–3 experiments were performed for each genotype; averages and SEMs are plotted; ANOVA repeated measures tests with Bonferroni post hoc tests were performed in R. ***p<0.001. DAT^{fmn} (obtained from Kazuhiko Kume, Nagoya City University, Japan).