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Genetics and neurobiology of aggression in Drosophila

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Aggressive behavior is widely present throughout the animal kingdom and is crucial to ensure survival and reproduction. Aggressive actions serve to acquire territory, food, or mates and in defense against predators or rivals; while in some species these behaviors are involved in establishing a social hierarchy. Aggression is a complex behavior, influenced by a broad range of genetic and environmental factors. Recent studies in Drosophila provide insight into the genetic basis and control of aggression. The state of the art on aggression in Drosophila and the many opportunities provided by this model organism to unravel the genetic and neurobiological basis of aggression are reviewed.

Introduction

Aggressive behavior is widely present throughout the animal kingdom and is crucial to ensure survival and reproduction. Aggressive actions are used to acquire territory, food, or mates and in defense of the individual or its progeny against predators or conspecific rivals. Additionally, in some species these behaviors are necessary to establish a social hierarchy. By contrast, excessive aggression implies risky and energy consuming acts, which can be evolutionary unfavorable.

Aggression is a complex behavior influenced by a broad range of genetic and environmental factors. Many of these factors, such as neurotransmitters, hormones, pheromones, sex and individual anatomical differences, have been studied in a variety of species.[1-19] These studies, however, often reported inconclusive or contradictory results both within and between species. Examples of divergent results within species include the role of testosterone and cortisol in vertebrate aggression and the differential effects of neurotransmitters on aggression in Drosophila (see below for a detailed discussion).[3] An example of a contradictory result between species is the opposite role for certain neurotransmitters between crustaceans and vertebrates.[3,4,8,16,20] Overall, these observations illustrate the complex regulatory mechanisms underlying this behavior.

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Recent studies in Drosophila provide insight into the genetic basis and control of complex behaviors, including aggression, and highlight the importance of interaction networks among many pleiotropic loci with relatively small effect sizes.[11,21-25] Thanks to the availability of numerous genetic resources, the ease to perform genetic manipulations and the possibility to control environmental influences as well as the genetic background, *Drosophila melanogaster* offers the opportunity to integrate single gene molecular genetics and whole genome quantitative genetics and thus unravel the genetic complexity and the neurological basis of this behavior.

Aggression in Drosophila

Agonistic behavior in general was first defined by Scott and Fredericson in 1951 as a continuum of behaviors from threat to aggression to submission.[26] The term aggression, however, has been much harder to define. In the context of social behaviors, aggression can be defined as species-specific behaviors associated with attack, and more broadly, threat.[27,28] This definition covers the parameters used to study this behavior in Drosophila.

Aggressive behavior in Drosophila was first observed in 1915 by Sturtevant, who reported males to spread their wings, run at each other, and apparently butt heads when courting the same female.[29] The first genetic study was the discovery of the involvement of the *ebony* gene in aggressive behavior.[30] In recent years, the continuously expanding collection of genetic tools in Drosophila has made it increasingly possible to study the genetic and neurobiological basis of aggression.

Inter-male aggression in Drosophila has been well described ethologically and shown to consist of behavioral modules which include both threat and attack behaviors.[31-35] In *Drosophila melanogaster*, these modules include: approaching, where one fly lowers his body and moves in the direction of the other; wing threats, where one fly quickly raises his wings toward its opponent; lunging, where one fly throws himself on his opponent; boxing, where both flies raise up on their hind legs and hit each other with their forelegs; tussling, where both flies tumble over each other; fencing or kicking; chasing and holding (see **Table 1**).[35] These aggression modules can form a continuous level of low aggression that can escalate to modules with high aggression, but they can also happen as isolated events.[14,35] Most of the fighting time is taken up by low intensity aggression such as fencing, while high intensity events such as lunging, boxing and

tussling have been reported to be rare in different Drosophila species.[35-37] Most research mainly focused on males because they display more aggression. However, female aggression has also been described in detail and shown to differ from male aggression both ethologically and motivationally. Part of the aggression behavioral repertoire is shared between males and females but some sex-specific behavioral modules occur (see **Table 1**).[5] Boxing and tussling, for instance, have been reported to be male-specific. Furthermore, while males fight both over food and females, females fight mainly over food resources, especially if these resources include yeast. It has been suggested that female aggression in *Drosophila melanogaster* relates mostly to reproductive behaviors such as egg laying which is enhanced by yeast and its metabolites.[5,38]

Drosophila male aggressive behavioral modules have been used to analyze two closely related social behaviors, dominance and territoriality. Dominance can be defined as having a higher social status than other individuals of the group and often implies "priority acquired by past or present aggressive behavior." However, in other species it was shown that aggression is not crucial to obtain and keep a dominant status. Dominance is a male-specific behavior that is stable over a certain amount of time.[39-46] Dominance is studied by analyzing the males that win or lose subsequent fights and involves flies remembering their previous opponents over a certain amount of time.[47,48]

Territoriality can be defined as occupancy of a defended area that is used exclusively by the defending individual.[39-41,49,50] Different studies have described the ecological circumstances

Table 1. Modules of aggressive behavior in male and female Drosophila

Male	Female
Retreat	
Walking, flying or running away	Walking or flying away
Approach/turn toward	
Walk toward the opponent while lowering body	Turn/walk toward the opponent
Wing threat	
Raise both wings to a 45° angle toward opponent (> 1s) Flicks wings at 45° angle while facing away from opponent	Raise one or both wings to a 45–90° angle toward opponent (< 1s)
Lunge	
Rear up on hind legs and collapse on opponent	Rear up on hind legs and collapse on opponent
Shove	
Not observed	Thrust the torso toward the opponent with both forelegs extended without recoil
Thrust with a wing threat	
Not observed	Thrust and lift one or both wings to a 45–90° angle (< 1s)
Head butt	
Not observed	Thrust the torso toward the opponent and strike the opponent with the head; usually followed by recoiling of the torso
Fencing	
Fencing (low): extend one leg and tap opponent's leg Fencing (high): extend leg forward and push opponent facing each other	Fencing (low): Extend leg and contact the opponent in a normal standing posture Fencing (high): Stand tall on the middle and rear legs and contact the opponent with the forelegs (can be combined with a wing threat < 1s) Fencing and feeding: Extend the middle or rear legs and contact the opponent while feeding Fencing threat: Extend the middle legs without contacting the opponent
Chasing	
Run after opponent	Not observed
Holding	
Grasp opponent with forelegs and try to immobilize	Not observed
Tussling	
Tumble over each other, sometimes leaving food surface	Not observed
Boxing	
Rear both up on hind legs and strike the opponent with forelegs	Not observed

Ethogram describing the observed modules of aggressive behavior in male and female Drosophila. The listed modules include threat, attack and retreat behaviors. Some of these modules are used by both sexes or show only subtle differences, while others are sex-specific.

under which Drosophila species exhibit territoriality and the evolutionary relevance of this behavior. In different Drosophila species, territoriality in a natural habitat has been shown to be closely linked with mating behavior. Males display territoriality in order to ensure the control over a resource for breeding and feeding. In highly territorial species, including the Hawaiian *Drosophila heteroneura* and *Drosophila silvestris*, this behavior even results in the aggregation of males in a lek in which these males defend their own leaf or part of rotten fruit.[51-54] *Drosophila melanogaster* displays much less territorial behavior than its related Hawaiian species. The few observations of *Drosophila melanogaster* under field conditions show that defense of territory is rare and that, contrary to *Drosophila pseudoobscura*, fighting in *Drosophila melanogaster* might be unimportant to acquire a mating advantage in the field.[54,55] The majority of the observations of *melanogaster* territoriality have been made in the lab.[30,31,56-59] Under these conditions, *Drosophila melanogaster* territoriality has been described as a conditional strategy, where flies would only invest in the defense of an area under certain conditions where territoriality could lead to a mating advantage. These would include presence of females or males, attractive food, but also the occurrence of only a small number of other males and only a relatively small area of food to defend. It seems that only under these conditions, the territorial males obtain a mating advantage.[60] Remarkably, in *Drosophila silvestris*, the highly territorial and aggressive Hawaiian species, mating success has not been linked with territoriality in a lab environment.[61]

These findings illustrate that although many characteristics of the behavioral repertoire of different insects have been reproduced in lab assays, the extrapolation to and the relevance of these data for a natural habitat need to be made with caution. A recent study of cricket behavior supports this notion. While dominant cricket males in the lab are capable of monopolizing access to females, in the wild these males seem to have many fewer mates than their subordinate opponents.[62] A number of factors that affect the outcome of aggression assays with *Drosophila melanogaster* have been identified. The observation of aggressive behaviors in a lab setting shows that the different Drosophila species have the innate capability to fight, but also illustrates that there are clear effects of the environment on the initiation and sequence of aggressive behaviors. It has been reported that the size of the arena in which the experiment takes places can have a significant influence on the behavior of the flies. Smaller arenas have been described to cause an unconditioned reflex reaction which leads to increased activity or arousal.[63] Furthermore, although these findings have not been analyzed further, an optimal arena size and shape have been proposed to induce aggression between Drosophila males while the lack of possibility for the losing male to escape can enhance aggression.[15,35,63] These influences of the size of the available territory on social strategy have also been described in other species. The pupfish *Cyprinodon variegates*, for instance, establishes a territorial breeding system in large tanks while small tanks lead to a dominance hierarchy, in which one male controls most of the oviposition sites and mates with most females.[64,65]

How to study aggression in Drosophila. In general, three main setups have been described to analyze aggression in Drosophila (**Fig. 1**). All three assays allow the observation of the aggression behavioral modules in males and females. Depending on the setup dominance or territoriality can also be analyzed. The various published studies are further distinguished by additional variables at the social level, such as the number of flies tested in the arena, their social experience and the presence or absence of females, or at the level of different aggression modules being measured. In some cases the focus was on actions that are exerted by a single fly, while in others the focus was on dyadic behaviors.[47,66] Some studies focus only on high intensity aggression modules which have been reported to constitute only a small portion of the fighting time but which lead to dominance. Other studies show the benefit of analyzing all aggressive modules to decipher the mechanisms underlying aggression. Different serotonin (5-HT) receptors, for instance, have been shown to alter different parts of the aggressive repertoire.[14] Opposite effects on lunging, tussling and chasing compared with wing threats were also observed upon genetic manipulation of cholinergic neurons.[67]

The first reported assays are variations on the same setup and make use of an arena with a centrally placed food cup to which yeast paste or a virgin female can be added.[5,7,14,15,68-71] Whereas in the original assay six males were used, in a simpler variant, aggressive encounters between two flies are recorded, usually during a 20–30 min period. This setup is compatible with CADABRA software, an application which allows the automated scoring of lunging, tussling, wing threats and chasing of eight pairs of flies simultaneously.[67] Next, an arena chamber was described that consists of a thick plexiglass plate with 35 evenly spaced cells, allowing the simultaneous recording of 35 arenas during a 15 min period.[37,72] Each arena contains two flies, but no food or virgin is introduced. Finally, a very simple assay, first used to describe altered aggression in *fruitless* (*fru*) mutant males, was described that allows the analysis of the aggressive encounters between groups of eight flies over a two-minute period.[6,12,33,73] This assay encompasses the instant scoring of the behavior, i.e., counting of all aggressive interactions among all eight males, without the need of analyzing recordings after the test period. This assay makes use of a standard food vial with a small drop of food.

Neurotransmitters and Aggression

Multiple neurotransmitters as well as the neuroactive peptide NPF have been shown to play a role in the processing of sensory information relevant to aggression and the generation of an appropriate aggressive response.[1,7,9,13-15,66,74] The effects of these neurotransmitters are often ambiguous and are likely to depend on multiple factors including the receptor subtypes involved, the necessity of intermediate levels of the neurotransmitter or the spatio-temporal activation pattern of the pathways (**Fig. 2**). Furthermore, some of the observed discrepancies could be due to the different assays or arenas used, the differences in the genetic background of the flies, the different ways of scoring aggressive behavior and the use of flies with different social experience.

Octopamine-tyramine. In the context of aggressive behavior, tyramine (TA) and octopamine (OA) have been the most extensively studied. Tyramine-β-hydroxylase (TβH), the enzyme

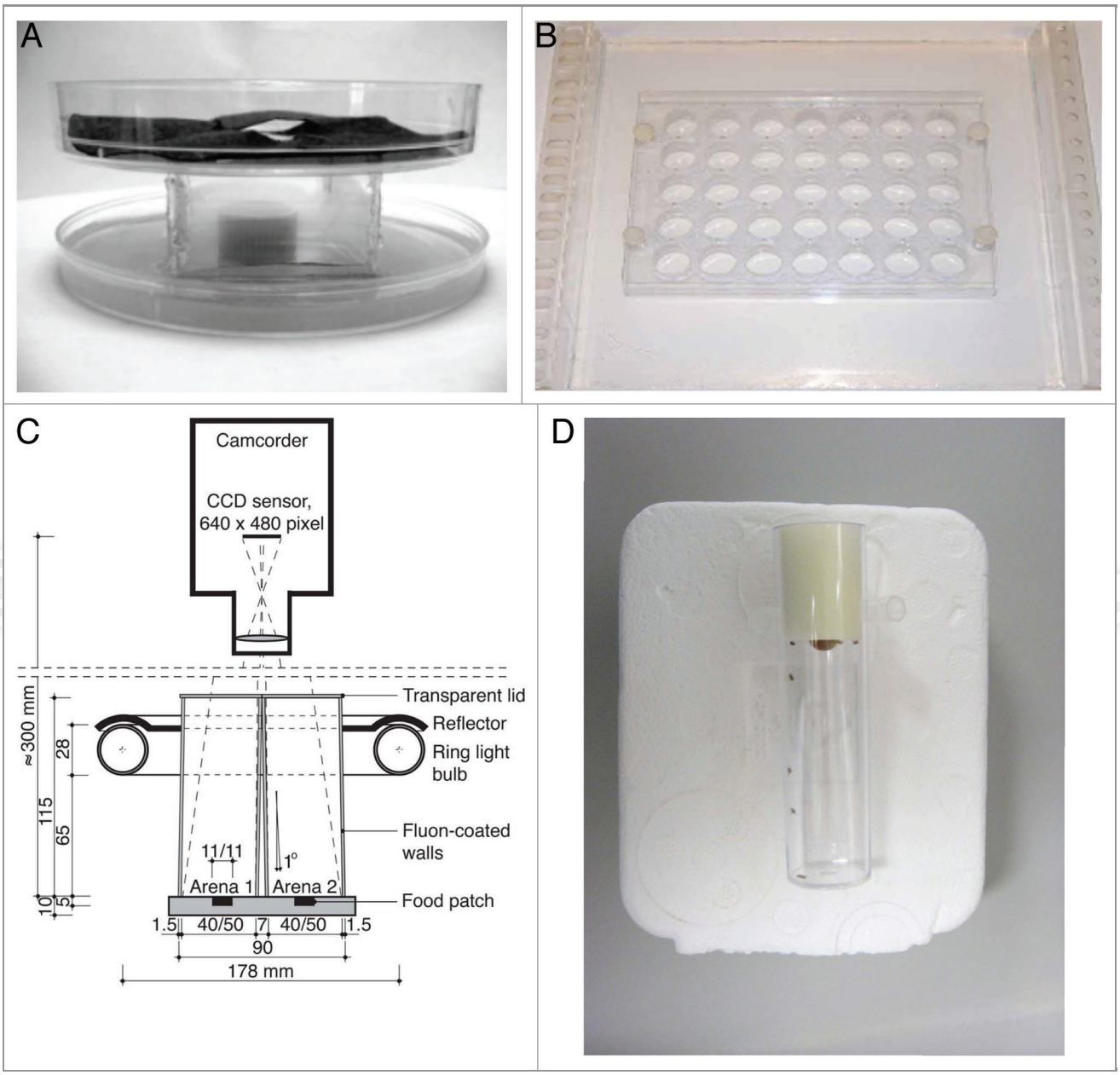

Figure 1. Different set-ups to study aggression in Drosophila. (A) Arena with a centrally placed food cup that can contain yeast paste or a virgin female, allowing the videotaping of aggressive behavior.[68] (B) Set-up allowing the simultaneous recording of 35 arenas, usually during a 15 min period.[72] (C) Scheme of a set-up compatible with CADABRA software allowing automated scoring of lunging, tussling, wing threats and chasing of two pairs of flies simultaneously. This set-up can be expanded to analyze eight pairs of flies simultaneously.[67] (D) Set-up allowing the instant analysis of the aggressive encounters between groups of eight flies over a 2 min period.[6,12,73]

responsible for the conversion of TA to OA, was one of the first enzymes shown to be involved in aggression in Drosophila. $T\beta H^{nM18}$ null mutants have undetectable levels of octopamine and a 10-fold increase in tyramine levels. $T\beta H^{nM18}$ males display decreased aggressive behavior, reduced transitions to aggressive behavior and decreased lunging, while females show prolonged fighting latency and reduced head butting. The magnitude of the effect seems to depend on the genetic background of the flies.[7,13,15,74]Interestingly, $T\beta H^{nM18}$ has also been associated with an increase in courtship behavior in socially naïve males, which could suggest a switch in behavioral choice between aggression or courtship.[74] However, this change in behavior could not be replicated.[13]

The changes in aggression in *TβH*-null mutants can in principle be due either to the decreases in OA or to the increase in TA. Flies have two tyrosine decarboxylase genes that convert tyrosine to TA, the non-neuronally expressed *Tdc1* and the neuronally expressed *Tdc2*. Male flies mutant for *Tdc2* ($Tdc2^{R054}$)

have no detectable levels of TA or OA in the brain and are less aggressive compared with controls.[15] Thus, it is the loss of OA that is responsible for the observed aggression phenotype.

The aggression modulating effect of OA has been further analyzed using pharmacological or genetic techniques. Pharmacological stimulation of OA signaling has been shown to enhance male aggression, but this effect is only present in socially experienced flies.[13,15] Two explanations for this observation have been proposed. First, OA might be important in the regulation of social experience, hence resetting aggression after social experience. Second, it might be impossible to detect higher aggression levels in already highly aggressive socially naïve flies.[13] However, it also cannot be excluded that the different results are due to experimental variations such as different arenas or differences in scored parameters.

Genetically induced changes in OA signaling were also shown to alter aggression. Ubiquitous *TβH* expression can induce elevated aggression in socially experienced wild-type flies but not in socially naïve flies.[13] However, it does seem to partially rescue the aggression phenotype of socially naïve $T\beta H^{nM18}$ null mutants.[15]

Neuronal activation of OA neurons in adults leads to the same phenotype of elevated aggression in socially experienced flies while acute silencing results in the opposite effect in socially naïve flies, indicating that the aggression phenotype in *TβH* mutants is not due to developmental defects.[13,15] Activation of OA neurons in socially naïve flies, however, does not affect aggressive behavior.[13,75]

In the Drosophila adult brain, there are approximately 100 octopaminergic neurons. An important question is then whether it is only specific subsets of these neurons that are required for aggressive behavior. Expression of TβH selectively in the Tdc2 circuit rescues the aggression phenotypes in both male and female hypoaggressive *TβH*-null mutants, thereby demonstrating the neuronal requirement of OA in the phenotype.[13] Further dissection of this circuit narrowed the relevant neurons down to a distinct subset of octopaminergic neurons in the subesophageal ganglion (SOG). In the SOG, a second subset of octopaminergic neurons in which OA colocalizes with FRU, another gene affecting aggression, have been proposed to play a role in the decision-making network that controls the shift between aggressive and courtship behavior.[74,75]

Serotonin. Initially, pharmacological studies reported that 5-HT does not have an influence on aggression in *Drosophila*.[7] More recently, it has been shown that drug-induced increases of 5-HT and overexpression of *Tryptophan hydroxylase* (*Trh*), the rate limiting enzyme in 5-HT synthesis increases aggression.[66] Consistent with this, selective activation of serotonergic neurons in the brain by means of *Trh -gal4* results in increased aggression with flies that escalate fights faster and with increased intensity.[9] By contrast, Dierick and Greenspan (2007) showed that pharmacological inhibition or silencing of 5-HT neurons has no effect on aggression. However, Alekseyenko et al. (2010) found that flies with acutely inhibited 5-HT neurons fight but do not escalate.[9] Overall, these results indicate that the effects of 5-HT are complex. The different effects of 5-HT might depend on the differential regulation of the behavior by different receptor types.[14] Drosophila expresses three types of 5-HT receptors; 5-HT2Dro, 5-HT1A-like and 5-HT7.[14] Using specific pharmacological modulation of these receptors, Johnson et al. showed that 5-HT2 and 5-HT1A-like receptors differentially regulate aggression in Drosophila. While activation of 5-HT2 receptors decreases overall aggression, activation of 5-HT1A-like receptors induces the opposite effect.[14] Both receptors influence different aspects of the behavior: 5-HT2 receptor manipulation primarily alters lunging and boxing, whereas 5-HT1A-like receptor manipulation primarily affects wing threats and fencing.[14]

Dopamine. Pharmacological alteration of DA levels in Drosophila suggests that the effects of DA on aggressive behavior are complex.[7] Spatiotemporal inactivation of neurons expressing *Dopa decarboxylase* (*Ddc*), the enzyme responsible for the final common step in 5-HT and DA biosynthesis, eliminated mid- and high-level aggression.[9] However, neither silencing serotonergic nor dopaminergic neurons individually mimics the phenotypes seen when both circuits are silenced simultaneously, suggesting that the interplay between both circuits is required for the regulation of aggression.[9] Recently, it has been shown that there are at least eight types of dopaminergic neurons.[76] This observation raises the possibility that there may be dopaminergic neuron subtype-specific effects on aggression.

Acetylcholine. In Drosophila, most sensory neurons and many central neurons are cholinergic. The effect of these neurons on aggressive behavior in Drosophila has not been extensively studied and the direct effects of alterations in neurotransmitter levels have not been investigated. Two independent publications report the effects of feminizing these neurons using *Choline acetyltransferase (Cha)-gal4* and UAS-*transformer* and show that this leads to an increase in specific aggressive actions such as lunging, boxing and chasing, whereas others such as wing threats are reportedly decreased.[1,67] The overall level of aggression, however, remains normal.[1] Although alterations in genes of the sex determination hierarchy have been reported to induce female fighting patterns in males and vice versa, feminized *Choline acetyltransferase* (*Cha*)-*gal4* ; UAS-*transformer* males show no changes in male fighting patterns. Instead, they show an increase in male-male courting behavior and an absence of male-female courting.[1,33,48,67] The reported effects seem to be due to a developmental effect with the phenocritical period in late larval to early pupal stages.[1] Given the large number of cholinergic neurons in the nervous system, it is unclear which subset(s) could be responsible for the observed behavioral changes and whether these differences in aggressive behavior relate to inappropriate sensory input arriving in the CNS or to changes within the CNS circuits themselves.[1] It has been argued that male specific cholinergic neurons, which express the male forms of fruitless or doublesex and represent 10% of the total number of cholinergic neurons, could play a major role, but this has not been investigated further.[1]

Neuronal Circuits Involved in Aggressive Behavior

Integration of sensory input. Aggressive behavior requires reception of sensory input followed by the interpretation and

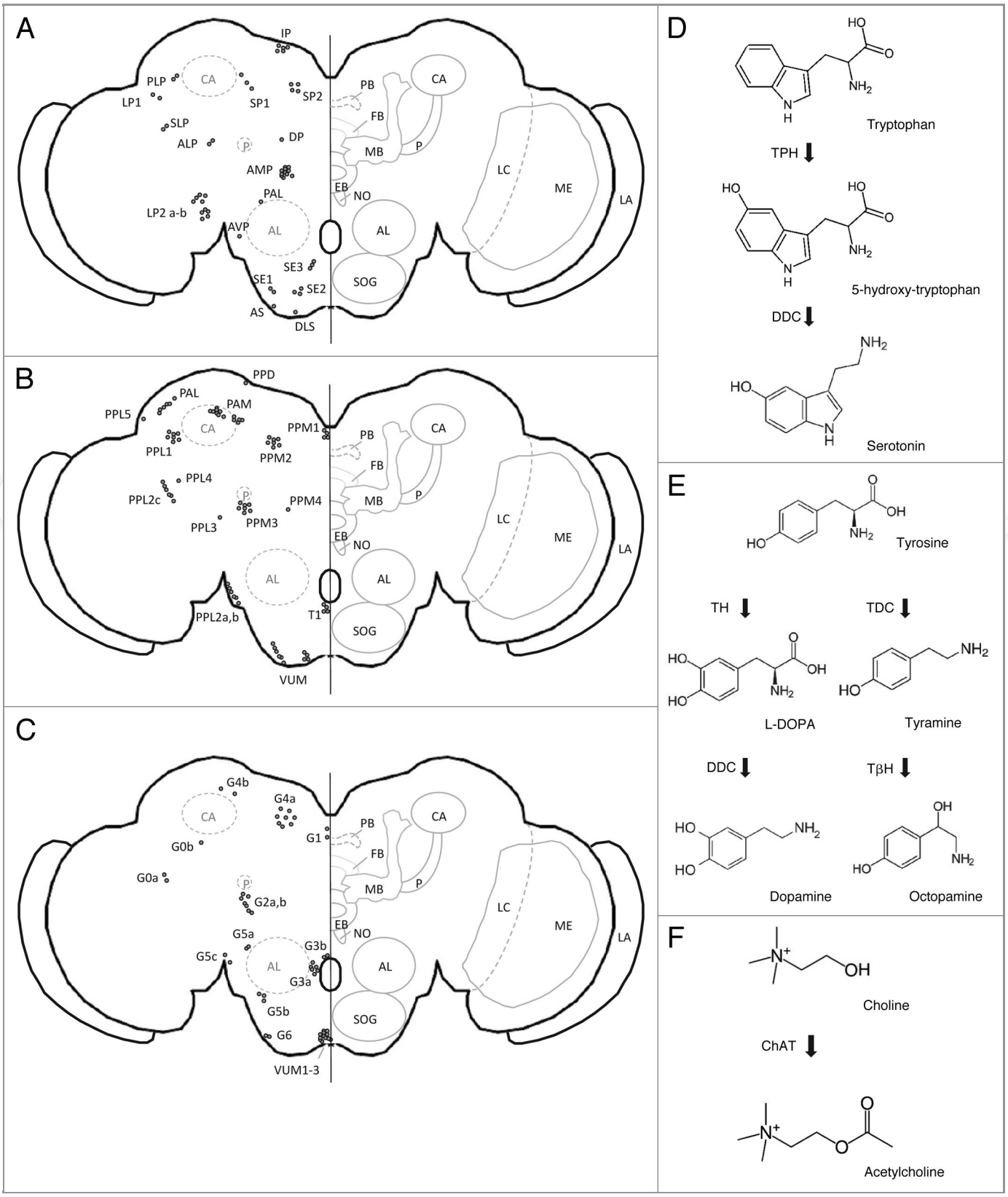

Figure 2. For figure legend, see page 41.

Figure 2 (See opposite page). (A–C) Schematic representation of adult central brain cells reported to be serotonergic, dopaminergic or octopaminergic. Cholinergic cells were reported to be widespread throughout the entire brain making it difficult to present a schematic overview.[140-143] PB, protocerebral bridge; FB, fan-shaped body; EB, ellipsoid body; NO, noduli; MB, mushroom bodies; P, mushroom body peduncle; CA, mushroom body calyx; AL, antennal lobe; SOG, subesophageal ganglion; LC, lobula complex; ME, medulla; LA, lamina. (A) Serotonergic cells. LLP1, posterior lateral protocerebrum; LP2a, between the medulla and the lateral protocerebrum; LP2b, between the medulla and the lateral protocerebrum; DP, dorsal protocerebrum; SP1, posterior to superior median protocerebrum; SP2, posterior median protocerebrum; IP, posterior inferior median protocerebrum; PLP, posterior lateral protocerebrum; SLP, superior lateral protocerebrum; AMP, anterior median protocerebrum; ALP, anterior lateral protocerebrum; AVP, anterior ventral protocerebrum; PAL, posterior to antennal lobe; DLS, dorsal lateral subesophageal ganglion; AS, anterior subesophageal ganglion; SE1, lateral subesophageal ganglion; SE2, anterior lateral subesophageal ganglion; SE3, most ventral subesophageal ganglion.[144-146] (B) Dopaminergic cells. PAM, dorsomedial anterior protocerebral; PAL, dorsolateral anterior protocerebral; PPM, dorsomedial posterior protocerebral; PPL1, dorsolateral posterior protocerebral; PPL2, lateral posterior protocerebral; PPD, protocerebral posterial dorsal; T1, tritocerebrum; VUM, ventral unpaired medial neurons.[76,147,148] (C) Octopaminergic cells (nomenclature according to Sinakevitch and Strausfeld, 2006; nomenclature between brackets according to Monastirioti et al., 1995). Cluster G0a, Cluster G0b (LP, lateral protocerebrum cell), Cluster G1 (DMC, dorsal medial cluster), Cluster G2a (~DAC, dorsal anterior cluster?), Cluster G2b (~DAC, dorsal anterior cluster?), Cluster G3a (AL, antennal lobe cluster), Cluster G3b, Cluster G4a (DPC, dorsal posterior cluster), Cluster G4b (DPC, dorsal posterior cluster), Cluster G5a, Cluster G5b,c, Cluster G6, VUM, Ventral unpaired median neurons 1–3 (SM, subesophageal medial). (D-F) Diagrams representing the biosynthetic pathways of acetylcholine and the monoaminergic neurotransmitters: serotonin, dopamine and octopamine. (D) Serotonin synthesis. TPH, Tryptophan hydroxylase; DDC, DOPA decarboxylase. (E) Dopamine/octopamine synthesis. TH, Tyrosine hydroxylase; TDC, Tyrosine decarboxylase; DDC, DOPA decarboxylase; TβH, Dopamine β hydroxylase (F) Acetylcholine synthesis. ChAT, Choline acetyltransferase

integration of these cues and the generation of an appropriate response. Differences in the development or function of the neuroanatomical structures mediating these processes can lead to the generation of an abnormal aggressive response to a certain cue (**Fig. 3**).

The generation of an aggressive response to external stimuli is influenced by many factors. One of these is the social history of the individual fly. Social experience influences aggression with socially naïve males being more aggressive.[13,67,77,78] About 200 genes have been shown to be differentially expressed between socially naïve and experienced flies.[79] Interestingly, one of these genes, *Cyp6a20*, is associated with pheromone sensing, suggesting that sensitivity to these pheromones provides a manner in which social experience modulates aggressive behavior.[79] In addition, memory of previous fights seemed to induce alterations in the fighting intensity among familiar male opponents.[47]

Pheromone signaling is the best studied sensory input system in the context of aggressive behavior.[10,77,80-82] Volatile pheromones are detected by the olfactory system, while non-volatile cuticular hydrocarbon pheromones signal via the gustatory system. Both have been reported to modulate aggressive behavior. The volatile pheromone cVA promotes aggression among males via *Or67d* expressing olfactory receptor neurons.[10] This pheromone also seems to mediate the aggression suppressing effect of group housing via *Or65a* expressing olfactory receptor neurons.[81]

The non-volatile sex-specific cuticular hydrocarbons produced by the oenocytes, play an important role in sex recognition and thus the appropriate behavioral response of males toward other males or females.[82] Masculinization of female oenocytes for instance elicits an aggressive response of males toward those females. The aggression promoting effect of cuticular hydrocarbons was further supported by the reduced levels of aggression between oenocyte- depleted males.[80] One of the most prominent cuticular hydrocarbons, (z)-7-tricosene, plays an important role in this aggression regulatory mechanism. This pheromone acts in a hierarchical manner with cVA through the activation of the Gr32a gustatory receptor where (z)-7-tricosene is required for the aggression promoting effect of cVA, but not vice versa.[80] Aside from pheromones, auditory and visual sensory systems have been reported in the context of aggression.[15,83]

Blind *norpA* and motion blind homozygous *ninaE* males perform significantly fewer lunges thereby implicating visual information in aggression.[15] The *white* gene is another gene that has been investigated in the context of vision and aggression. Mutations in this gene lead to visual abnormalities, especially at high light intensities, but these flies are not blind. They have normal phototactive responses and show responses to light stimuli on ERG that are stronger due to the lack of light buffering by pigmentation.[84,85] *White* mutants show different behavioral abnormalities, but these could also be due to effects of this ABC-transporter in central brain structures. Indeed, for some behaviors it has been described that the effects in central brain structures can be independent from the effects on eye pigmentation, possibly due to alterations in different monoamine levels in the brain.[85-90] The effect on aggression of the w^{1118} allele, a null allele of *white* with a deletion at the 5' of the gene that includes exon 1, has been examined in different assays by different groups. One report did not show a significant alteration in aggression in these mutants, whereas other groups showed a significant reduction in lunging as well as other high intensity fighting and an increase in fighting latency.[9,13,15,91,92] Furthermore, an eye specific RNAi knockdown of the *white* gene using *GMR*-gal4 has also been shown to cause a significant reduction in lunging.[15] However, the effect of w^{1118} on lunging does not seem to be solely due to the effects in the eye.[15] Overexpression of *white* in the eye using *GMR*-gal4 in a w^{1118} mutant only partially rescues the lunging phenotype and *white* RNAi using various drivers expressed throughout the brain lead to significant decreases in this behavior.[15] In summary, the possible role of the *white* gene in aggression seems complex and not fully understood.

Auditory signals have been proposed to play a role in the recognition of an opponent in *Drosophila melanogaster* males.[83] Sound production during aggression seems to be a male specific trait.[83] Males produce acoustic signals during aggressive encounters that differ from courtship sounds. These signals mainly occur

upon tapping by an opponent and seem to be a recognition reaction by the tapped male when he finds out that his opponent is also a male, thus inducing an aggressive reaction. Further observations supporting the aggressive nature of these sounds are that they are capable of inducing retreat of the opponent and that they are only produced in certain situations. Retreat of the opponent, for instance, is solely accompanied by silent wing movements.

Neural circuits and aggression. Different central brain structures and neuronal populations play a role in the integration and interpretation of stimuli and in the generation of a behavioral response. However, only a subset of these structures has been more closely investigated in the context of aggressive behavior.

Olfactory information is transmitted along olfactory sensory neurons toward the glomeruli of the antennal lobe. In the antennal lobe, peripheral olfactory receptor neurons are interconnected by local interneurons involved in the local processing of olfactory information. This interconnectivity has been suggested to modulate the interplay between cVA signaling via the aggression promoting *Or67d* receptor and the aggression suppressing *Or65a* receptor.[81] The ORNs also connect to projection neurons which forward information toward the mushroom bodies and the lateral horn. The latter mediates innate behavioral responses to odors.[93,94] The projection neurons that transmit the information of the male-specific, aggression-mediating pheromone cVA toward the lateral horn are FRU positive and show sexual dimorphism, suggesting that these neurons may modulate aggressive behavior.[95]

Gustatory receptor information on the other hand is primarily transmitted via gustatory receptor neurons toward the subesophageal ganglion. How this information is further processed in the brain is less well understood, but subesophageal ganglion neurons project toward multiple regions in the brain, including the antennal lobe, the lateral horn and the mushroom bodies, which could present a way to relay gustatory and contact pheromone sensory data to different higher processing centers in the brain.[74,96]

Visual information passes through multiple well-studied layers in the optic lobes. The central integration of this information, however, is less well known. The mushroom bodies and the ellipsoid body as well as different neurons in the lateral protocerebrum have been implicated in certain forms of visual learning.[97-99]

Auditory information is transmitted by neurons of the Johnston's organ that innervate the antennal mechanosensory and motor center of the brain, a neuropil lateral to the subesophageal ganglion and antennal lobes.[100] It is unknown how this information is further integrated in the brain.

One of the main higher integration sites in the brain, the mushroom bodies, are involved in the integration of many of the aforementioned sensory cues. Mushroom bodies have been shown to play a key role in multiple behaviors such as olfactory information processing, memory formation, sleep, the higher control of locomotion, and the processing of visual context information.[99,101-104]The mushroom bodies have also been implicated in aggression.[6,7,11,12] Blocking their synaptic output results in the abolishment of aggressive behavior.[7] Many pleiotropic genes influencing aggression also function in mushroom body development suggesting a relation between brain development and aggressive behavior in adult flies.[6,11,12] The length of the α lobes of the mushroom bodies has been correlated with aggressive behavior in viable P-element insertion mutants.[11] The significance of this observation is unclear. However, a relationship between mushroom body volume and aggression has also been described in two paperwasp species, *Polistes instabilis* and *Mischocyttarus mastigophorus,* suggesting there could be conserved roles involving mushroom body structure and its plasticity in insect aggression.[105,106]

Finally, the *fruitless (fru)* circuit has been shown to play an important role in the regulation of sexually dimorphic responses to sensory cues. *fru*, known for its prominent role in male courtship behavior undergoes sex-specific splicing, and it is involved in the sex determination hierarchy. Alterations in splicing of this gene are sufficient to elicit female fighting patterns in males and vice versa.[33,48] Subgroups of *fru* neurons are involved in the control of these sexually dimorphic patterns.[107] Specific *fru*-positive octopaminergic neurons in the subesophageal ganglion, for example, have been implicated in the decision between aggressive or courtship behavior.[75] The subesophageal ganglion plays a role in pheromone recognition in addition to its better-known function in taste processing. The role of the subesophageal ganglion in aggression is further supported by the presence therein of other, non-*fru*-positive, octopaminergic neurons that also modulate aggression.[13]

Due to the complexity of aggressive behavior and the vast connectivity pattern throughout the brain of the neuropils known to be involved in aggression, it is expected that many more neuronal populations will exert an influence on this behavior.

Pleiotropic Networks of Many Interacting Genes

In the 1990s, the first attempts to map human loci involved in complex behavioral traits using the available genome-wide markers identified a limited number of loci with a large effect size.[108-112] However, novel high resolution mapping in model organisms and genome wide association studies in patients suffering from disorders of which aggression represents an endophenotype, revealed that the genetic effect sizes for common variation are a lot smaller than previously expected.[109,113,114] These studies showed the involvement of a large number of genes and the potentially high importance of rare variants in the control of these complex behaviors.

In Drosophila, quantitative analyses demonstrated that investigating genes involved in biologically likely pathways only partly identify the genetic network and the neuronal populations that regulate this behavior.[11,12,23,37,73,115] In effect, currently available evidence reveals that aggressive behavior in Drosophila is controlled by (a) complex genetic network(s) involving a large number of pleiotropic and epistatically interacting genes. The existence of such an elaborate network controlling aggression is not surprising given the genetic architecture of other complex traits in Drosophila and other organisms and given the number of neurobiological processes that have been shown to influence

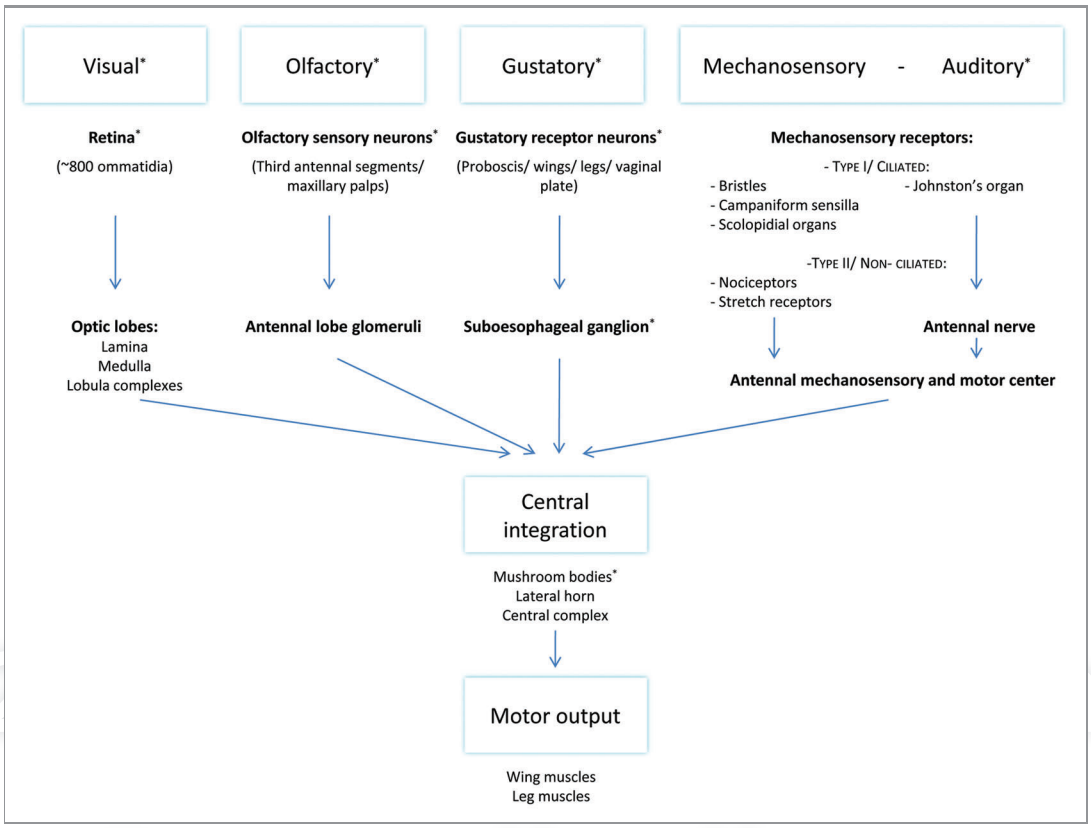

Figure 3. Scheme representing the flow of information provided by external stimuli to integration centers in the central brain. This central integration results in interpretation and integration of the different inputs and the generation of an appropriate response. The role of stimuli or neuronal structures marked with an asterisk (*) in aggression has been more closely analyzed. Visual information is received by the retinal ommatidia and travels through the different layers of the optic lobes, lamina, medulla and lobula complexes, to the central brain.[149] The described higher integration centers of this visual information include the mushroom bodies, the central complex and different neurons in the lateral protocerebrum.[97-99,150] Olfactory information is sensed by olfactory neurons expressing olfactory receptors located on the third antennal segments and the maxillary palps. Odorant cues travel via these neurons to the glomeruli in the antennal lobe from where projection neurons send this information to higher integration centers, including the mushroom bodies and the lateral horn.[93,151-155] Gustatory signals are sensed by gustatory receptors expressing gustatory receptors located on the proboscis, wings, legs and vaginal plate. These neurons all project to the SOG. The SOG has been shown to project toward multiple other brain regions including the antennal lobe, the lateral horn and the mushroom bodies.[96,155,156] Mechanosensory information is sensed by a variety of receptors, located all over the body, which can be subdivided into a ciliated and a non-ciliated group. Non-ciliated mechanosensory receptors include nociceptors and muscle and visceral stretch receptors. Ciliated mechanosensory receptors include: bristles responsible for touch perception, campaniform sensilla on wings and haltere providing info on flight parameters and chordotonal organs including scolopidial organs providing proprioceptive and gravireceptive information.[157,158] The fly's largest chordotonal organ, Johnston's organ, is located in the second antennal segment and represents the flies ear. Part of these neurons have been shown to innervate the antennal mechanosensory and motor center of the brain, a neuropil lateral to the SOG and the antennal lobes, further higher integration is mainly unknown.[100,157,158]

aggression in Drosophila itself.[21,109,116-120] Furthermore, it also makes sense from an evolutionary and biological point of view. As previously discussed, complex behaviors rely on the perception and integration of many layers of information as well as the capability to execute the behavior. Thus they depend on a vast number of biological processes, each characterized by their own (sub)network of genes and each under different evolutionary pressures.

Two independent studies that investigated the effects of artificial selection for aggressive behavior on transcript abundance provided a first insight into the overall genetic network underlying this trait. Dierick et al. identified 80 differentially expressed transcripts, while Edwards et al. found 1,539 altered transcripts.[37,73] Both experiments illustrate the possible role in aggression of transcripts involved in a wide variety of biological processes and molecular functions. A possible explanation for the difference in gene numbers between both groups is that genetic variation in the fly stocks (laboratory stock vs. recently derived from nature) that were used to initiate the selection experiments was different.

It is important to consider that transcriptional alterations do not provide information about the causality of the identified genes for the behavioral trait. However, for many of the transcripts it has been shown that these genes affect aggressive behavior. A screen of *P[GT1]* insertion lines, which was enriched for candidate aggression genes identified by the selection experiment of Edwards et al. (2006), showed direct effects on this behavior of mutations in 59 genes. Mutations in *Cyp6a20,* a gene identified

by Dierick et al. directly alters aggression levels.[37,121] Interestingly, this gene is involved in the regulation of behavioral differences between socially naïve and experienced flies.[79]

These studies also illustrate the extensive pleiotropy of the identified genes. Many of these genes show clear effects on other behaviors such as resistance to starvation stress, sleep and olfactory and locomotion behavior.[21,116,120,122] Others play a role in the correct development of sensory bristles and neuropils, such as the mushroom bodies and the central complex.[6,12,123] A detailed analysis of the *neuralized* gene revealed that alternative splicing can provide a molecular mechanism which forms the functional basis of the phenotypic pleiotropy.[6]

Analyses of the genetic networks underlying other complex behaviors such as olfactory avoidance behavior and startle induced locomotion showed that the corresponding genes often interact in a non-additive manner.[21,116] Therefore, it is not unexpected that epistasis would also be present among the genes that form the genetic basis of aggression. A first indication came from the mapping of aggression QTLs which are characterized by epistasis.[115] Furthermore, the analysis of the variation in aggressive behavior among 40 wild-derived inbred lines provided more evidence for the presence of non-additive interactions among the genes involved.[23] Recently, the analysis of the epistatic interactions between a set of ten hyperaggressive *P[GT1]* insertion lines provided the first insights into the complex nature of these interactions and their widespread influences on transcript abundance.[11]

Further analysis of the transcriptional network underlying aggression in the 40 wild-derived inbred lines identified networks of coregulated genes that are involved in this behavior.[23] In this analysis, the genetic network associated with natural variation in aggressive behavior was mapped by investigating the associations between aggression and quantitative trait transcripts (QTT) and single feature polymorphisms (SFP) and subsequently grouping the associated transcripts into genetically correlated modules. Two hundred sixty-six candidate aggression genes were identified. While mutations in some of the identified genes were shown to have a clear effect on aggression or have been shown to have an important role in this behavior, e.g., members of the Cytochrome P450 gene family, the identified genes showed only a small overlap with previously identified genes. However, they were part of nine distinct modules of genetically intercorrelated transcripts enriched for gene ontology categories previously implicated in aggressive behavior, such as neurodevelopment, visual perception and metabolic functions and male-biased transcripts.

The difference between the genes identified by the selection experiments, the QTL mapping and the transcriptional analysis of the inbred lines as well as the absence of some of the previously identified genes involved in aggressive behavior could be attributed to multiple factors.[23,37,73,115] First of all, as these experiments, with exception of the QTL mapping, are all based on expression analyses, the candidate aggression genes would not be identified if they are not genetically variable at the transcript level or if their transcripts do not vary or are only expressed at low levels during the analyzed developmental stage.[23,37,73] As the genome sequence of the 40 inbred lines will be available in the near future, it will be interesting to see whether polymorphisms at the DNA level linked with aggression will identify these previously known aggression genes. Furthermore, all of the described whole genome analyses, i.e., both selection experiments, the QTL mapping experiment and the transcript analysis of the 40 inbred lines, use different parental lines as a starting point.[23,37,73,115] As we already pointed out, epistasis plays an important role in controlling behaviors, thus a different genetic context, resulting from the different parental lines could have a major influence. Furthermore, it could be that the genetic variation captured in each sample was different, as would happen, for example, if the genetic basis of natural variation in aggressive behavior involves many different rare alleles with small effects. Finally, the known candidate genes, such as *fru*, are often involved in other important processes. This could make them subject to strong purifying selection and thus might not allow functional variation.[23]

The transcript analyses of the 40 inbred lines provides the first attempt to generate an overview of transcriptional modules that control aggression and could form the starting point for a systems genetics analysis of natural variation in this behavior. Such an approach aims to integrate the different layers of biological information between DNA and observed phenotype, consisting of RNA, proteins and metabolites.[124] In the near future, the availability of genome sequences of these 40 lines, and of in total 192 lines will give the opportunity to fill in another layer of this network, creating the possibility to directly link polymorphism to alterations in the coexpression network that result in behavioral changes.[23,125]

Perspectives

The genetic architecture of behaviors, such as aggression, is shaped by many interacting genes with pleiotropic effects. Quantitative genetic whole genome analyses enable more insight into the overall genetic networks and the molecular context that are at the basis of these behaviors. However, it is the combination of complex quantitative analyses with single gene molecular genetics that will allow the definition of the exact molecular functions of subsets of genes in this network.

The study of aggression in Drosophila using such integrative approach will lead to a better understanding of its genetic and neurobiological basis and the identification and characterization of neural networks that mediate or influence this behavior. We surmise that these insights will contribute to our understanding of the evolution of aggressive behavior and of the genetic basis of aggression in other species, including humans.

In Drosophila, territorial aggression has been observed in different species. *Drosophila hawaiensis* males, for instance, have been shown to vigorously defend their mating territory.[126,127] Analysis of differences in the genetic background between closely related species could help to understand the evolutionary forces leading to divergent aggressive behavior. In addition, the analysis of sequence variation and its functional consequences for genes involved in aggressive behavior in *Drosophila melanogaster* represents another avenue to study the genetics and evolution of aggression in other species. Supporting this contention, many

factors involved in the control of aggression seem to be conserved among insects. Experience and chemical cues influence this behavior, while neurotransmitters implicated in Drosophila aggression also mediate this behavior in ants and crickets.[128-136] Furthermore, QTL mapping revealed, besides neurodevelopmental genes and GPCRs, the metabotropic GABA-B-R1 receptor as a candidate aggression gene in honey bees.[137,138] Not only neurotransmitters and receptors appear to play comparable roles in insect aggression, but also the same brain structures mediate aggressive behavior. Similar to Drosophila, the mushroom bodies have been implicated in aggression in two paperwasp species, *Polistes instabilis* and *Mischocyttarus mastigophorus*.[105,106] The role of mushroom bodies is also supported by the observation that agonistic behavior in crickets is accompanied by the induction of c-Fos/FRA-like expression in the mushroom body neuropil.[139] It will be interesting and important to determine the extent to which the molecular networks underlying aggression are conserved between vertebrates and insects and whether this conservation is mainly situated at the gene level, with common functions in behavioral regulation between homologs, or at the systems level, where networks affecting shared biological processes lead to behavioral alterations.

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