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REVIEW

Evolution, developmental expression and function of odorant receptors in insects

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ABSTRACT

Animals rely on their chemosensory system to discriminate among a very large number of attractive or repulsive chemical cues in the environment, which is essential to respond with proper action. The olfactory sensory systems in insects share significant similarities with those of vertebrates, although they also exhibit dramatic differences, such as the molecular nature of the odorant receptors (ORs): insect ORs function as heteromeric ion channels with a common Orco subunit, unlike the G-protein-coupled olfactory receptors found in vertebrates. Remarkable progress has recently been made in understanding the evolution, development and function of insect odorant receptor neurons (ORNs). These studies have uncovered the diversity of olfactory sensory systems among insect species, including in eusocial insects that rely extensively on olfactory sensing of pheromones for social communication. However, further studies, notably functional analyses, are needed to improve our understanding of the origins of the Orco–OR system, the mechanisms of ORN fate determination, and the extraordinary diversity of behavioral responses to chemical cues.

KEY WORDS: Evolution, Development, Diversity, Olfaction, Odorant Receptor, Orco, Ant, *Drosophila*

Introduction

Animals rely on their chemosensory system to sense and discriminate among a large variety of chemicals in the environment, including general odorants for food source, pheromones for interactions with conspecifics, and repulsive cues from prey or toxic compounds. In insects, chemosensory neurons utilize a variety of receptor molecules to detect odors, including odorant receptors (ORs), gustatory receptors (GRs), ionotropic receptors (IRs) and other receptors, such as Pickpocket (PPK) and TRP receptors (Joseph and Carlson, 2015). Among them, the neurons specialized in detecting volatile chemicals (odorant receptor neurons, or ORNs, a.k.a. olfactory sensory neurons, OSNs) normally express ORs, although exceptions exist, such as CO_2 which is recognized by GR-expressing ORNs (Jones et al., 2007).

To discriminate a large amount of odorants, both insects and vertebrates utilize a decoding strategy of 'one neuron, one receptor': each ORN normally expresses only one *Or* gene, and all the ORNs that express the same *Or* gene project their axons to the same glomeruli in the antennal lobe of insects or the olfactory bulb of vertebrates, where they form synapses with higher-order neurons (projection neurons, or PNs, and local neurons, or LNs, for insects) (Komiyama and Luo, 2006). The regulatory mechanisms underlying the singular expression pattern of *Or* genes in ORNs are relatively well understood in *Drosophila*, a common and powerful insect model system. However, recent studies suggest that other insects may use different mechanisms for ORN development and *Or* choice (Trible et al., 2017; Yan et al., 2017).

Or genes have evolved multiple times and have different origins in insects and vertebrates (Hansson and Stensmyr, 2011; Robertson, 2019). In vertebrates, all ORs are G-protein-coupled receptors (GPCRs). Each GPCR contains seven transmembrane domains, an extracellular N-terminus and an intracellular C-terminus. In contrast, the insect *Or* gene family evolved from an ancestral *Gr* gene family (Brand et al., 2018; Robertson, 2019). Although insect ORs also contain seven transmembrane domains, they are not GPCRs but instead function as ion channels; they possess an inverted topology with intracellular N-termini and extracellular C-termini. Insect ORs appear to be heterotetramers likely composed of two copies of an obligate and common co-receptor called Orco and two copies of a ligand-binding tuning OR, which together form ligand-gated ion channels (Benton et al., 2006; Butterwick et al., 2018; Sato et al., 2008; Wicher et al., 2008). Normally there is only one *orco* gene in each insect genome and Orco sequences are highly conserved among insects. However, the number of *Or* genes varies from three in Odonata (including damselflies and dragonflies) (Brand et al., 2018) to 300–500 in ants (Fig. 1) (McKenzie and Kronauer, 2018; Opachaloemphan et al., 2018; Zhou et al., 2012). In hymenopteran eusocial insects, including wasps, bees and ants, *Or* genes form a large and highly divergent gene family. Among them, the '9-exon' *Or* gene subfamily is extensively amplified (McKenzie et al., 2016; McKenzie and Kronauer, 2018; Zhou et al., 2015, 2012). Interestingly, 9-exon ORs appear to be involved in recognizing and discriminating cuticular hydrocarbon (CHC) pheromones in hymenopterans (Pask et al., 2017).

In this article, we summarize the recent progress in understanding evolution, development and functions of odorant sensory systems in insects. With its powerful genetic tools, *Drosophila* provides a unique system to study the molecular mechanisms underlying ORN development. However, some mechanisms appear to not be fully conserved in other insects. Further investigations are required to explore the diversity of ORN development and function among insects.

Evolution and structure of odorant receptors in insects

The origin of ORs and Orco in insects has been extensively studied. *Or* genes have not been identified in non-insect arthropods, suggesting that these animals mainly rely on IRs and/or GRs for chemosensation. Indeed, in Crustacea, a single *Gr* and 108 *Ir* genes

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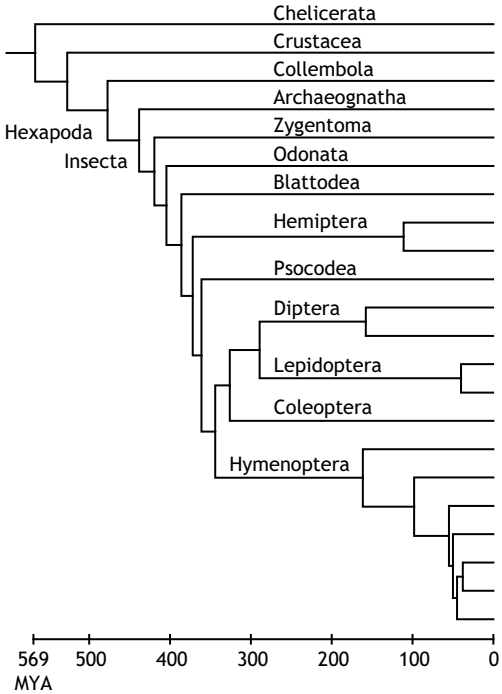
List of abbreviations

APF	after puparium formation
CHC	cuticular hydrocarbon
GPCR	G-protein-coupled receptor
GR	gustatory receptor
IR	ionotropic receptor
LN	local neuron
OR	odorant receptor
ORN	odorant receptor neuron
PN	projection neuron
SOP	sensory organ precursor

have been identified in the genome of spiny lobster, *Panulirus argus* (Kozma et al., 2018), and IRs are widely expressed in their chemosensory organs (Corey et al., 2013). In Chelicerata, the genome of the nocturnal wandering hunter spider *Dysdera silvatica* contains 12 *Gr* and 127 *Ir* genes (Vizueta et al., 2017). The non-insect hexapods, such as springtails and two-pronged bristletails, do not have *orco* and *Or* genes, while early-branching insects, such as jumping bristletails, contain five *Or* genes, but no *orco* gene (Brand et al., 2018). *orco* appeared in Zygentoma, an insect order that includes silverfish and firebrats. From that point (~440 MYA; Fig. 1), a single, highly conserved *orco* gene is present in every insect genome, while *Or* genes vary in number from three in early-branching insects to 300–500 in ants, with no clear orthology between receptors in different orders (Robertson, 2019). Additionally, Hymenoptera – ants, bees and wasps – display lineage-specific expansion of different subfamilies of ORs. Even in ants, the number of 9-exon ORs (the largest subfamily, see below) differs dramatically between species, suggesting highly diverse patterns of gene gain and loss among odorant receptors (Zhou et al., 2015, 2012). The rapid evolution of the OR gene repertoire may have facilitated the adaptation of insects to ever-changing environments and the origination of evolutionary novelties (e.g. eusociality).

Mounting evidence suggests that the molecular evolution of ORs likely led to adaptation to novel food resources or pheromone perception. Two dipteran species, *Drosophila melanogaster* and *Anopheles gambiae* (mosquitos), display differential sensing abilities to fruit esters versus human volatiles (e.g. aromatics), consistent with their ecological niche (Carey et al., 2010). In ants, sometimes closely related ORs display different ligand binding (tuning) profiles, as evidenced by functional characterization of ORs (Pask et al., 2017). For example, although *Harpegnathos* OR263 and OR348 share 93% protein sequence identity, only the former shows a strong response to the queen pheromone (Pask et al., 2017).

Most insect species have fewer than 100 *Or* genes. However, a very large expansion has been found in Hymenoptera, which contains many clades of eusocial insects, including ants, bees and social wasps. As *Or* genes have already expanded in solitary wasps, such as *Nasonia vitripennis* (225 *Or* genes; Fig. 1), it is conceivable that *Or* gene expansion was involved in the adaptation to certain environments, perhaps host recognition in solitary wasps. The expansion was later pre-adaptive to social evolution and further expansion in ants may have facilitated the recognition of complex social cues, including CHC pheromones for caste determination and nestmate versus non-nestmate discrimination (Fig. 1) (Carlin and Hölldobler, 1983; Morel et al., 1988; Ozaki et al., 2005). However, termites, which are non-hymenopteran eusocial insects in the order of Blattodea, do not show dramatic expansion of the *Or* gene family,



	Or	*orco*	*Gr*	*Ir*
Dysdera silvatica (spider)	0	0	12	127
Panulirus argus (lobster)	0	0	1	108
Tetrodontophora bielanensis (springtail)	0	0	NA	NA
Machilis hrabei (bristletail)	5	0	NA	NA
Thermobia domestica (firebrat)	43	1	9	19
Ladona fulva (dragonfly)	3	1	NA	NA
Zootermopsis nevadensis (termite)	63	1	80	150
Cyrtorhinus lividipennis (plant bug)	15	1	3	6
Gerris buenoi (water strider)	155	1	60	45
Pediculus humanus (human body louse)	10	1	6	10
Drosophila melanogaster (fruit fly)	60	1	60	64
Anopheles gambiae (mosquito)	79	1	76	46
Bombyx mori (silkmoth)	48	1	65	18
Manduca sexta (moth)	64	1	2	6
Tribolium castaneum (flour beetle)	261	1	215	71
Nasonia vitripennis (parasitoid wasp)	225	1	47	10
Apis mellifera (honey bee)	165	1	12	21
Harpegnathos saltator (Indian jumping ant)	347	1	17	23
Ooceraea biroi (clonal raider ant)	503	1	28	26
Solenopsis invicta (fire ant)	333	1	219	NA
Acromyrmex echinatior (leafcutter ant)	375	1	116	NA
Camponotus floridanus (carpenter ant)	352	1	46	31

Fig. 1. Evolution of chemosensory receptor genes in arthropods. The evolutionary relationships and divergence times among representative arthropod species are shown on the left; the cladogram is adapted from a recent phylogenomic analysis in insects (Misof et al., 2014). The number of chemosensory receptor genes (*Or*, odorant receptor; *orco*, odorant receptor co-receptor; *Gr*, gustatory receptor; *Ir*, ionotropic receptor) reported in each species is shown on the right, based on previous work (Armisen et al., 2018; Brand et al., 2018; Carey and Carlson, 2011; Croset et al., 2010; Hill et al., 2002; Howlett et al., 2012; McKenzie and Kronauer, 2018; Missbach et al., 2014; Oxley et al., 2014; Robertson, 2019; Terrapon et al., 2014; Wang et al., 2018; Wanner et al., 2007a; Wanner and Robertson, 2008; Zhou et al., 2015, 2012). Of note, in the water strider *Gerris buenoi*, 60 *Gr* genes encode 135 GR proteins via alternative splicing (Armisen et al., 2018).

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but rather an expansion of the *Ir* gene family (Terrapon et al., 2014), suggesting that chemosensory systems have evolved independently in various orders to achieve an advanced form of eusociality.

Another interesting example of non-eusocial expansion of *Or* genes is provided by the flour beetle *Tribolium castaneum* (261 *Or* genes; Fig. 1) (Robertson, 2019; Richards et al., 2008) and water strider *Gerris buenoi* (155 *Or* genes; Fig. 1) (Armisén et al., 2018). This *Or* gene expansion in some Coleoptera and Hemiptera species highlights other good systems, in addition to Hymenoptera, for analyzing ORN development. As described below, olfactory neural plasticity, i.e. Orco-dependent ORN development/survival, has been recently discovered in ants. It will be interesting to analyze whether the same regulatory mechanisms are also utilized in *Nasonia*, *Tribolium* and *Gerris*, where the *Or* gene family has also expanded.

While the *Or* gene family is enlarged in Hymenoptera, gene gains and losses are highly diverse in wasps, bees and different lineages of ants (Zhou et al., 2015). Positive selection has been found in ancestral branches of eusocial species, including solitary wasps and bees, suggesting that evolution of ORs is pre-adaptive to the evolution of eusociality. In addition, evolutionary gains or losses of *Or* genes are likely involved in the evolution of different patterns of social organization. The fire ant *Solenopsis invicta* contains a social chromosome, a non-recombining region with two distinct haplotypes (SB and Sb) that determine the reproductive caste pattern: monogyny (single queen) versus polygyny (multiple queens) (Wang et al., 2013). The heterozygous SB/Sb workers are normally found in polygynous colonies and do not accept monogynous queens, while homozygous SB/SB workers do not accept polygynous queens. A recent comparative analysis between SB and Sb workers identified two SB-specific *Or* genes, suggesting that loss of *Or* genes in the Sb workers may be involved in the differential discrimination between the two types of social organization (Cohanim et al., 2018). Fire ants have undergone an expansion not only of *Or* genes but also of *Gr* genes (219 *Gr* genes; Fig. 1), with a striking increase in the 'E' subfamily of *Gr* genes from none or only one gene in most insect species to 79 genes in fire ants (Zhou et al., 2015). Functional analyses will be required to further understand the role of species-specific gains or losses of chemosensory genes (see below).

Structure of OR complexes and function of Orco

Ever since the Orco–OR complex was characterized as a heteromultimer (Benton et al., 2006) that acts as a ligand-gated ion channel (Sato et al., 2008; Wicher et al., 2008), scientists have been eager to determine the structure of this complex. Recently, the cryo-EM structure of Orco from the parasitic wasp *Apocrypta bakeri* was shown to be a homotetramer (Fig. 2) (Butterwick et al., 2018), which allows spontaneous opening of a non-specific cation channel (Dobritsa et al., 2003; Jones et al., 2011; Pask et al., 2011). Consistent with this notion, *orco* mutant flies show strongly diminished spontaneous activity and odorant response in their ORNs (Larsson et al., 2004). The pore of the ion channel is formed by transmembrane helical structures from each subunit that interact via their conserved C-termini (transmembrane domains 4–7), which resembles acid-sensing ion channels and ATP-gated P2X channels (Butterwick et al., 2018). This conserved anchor domain appears to facilitate subunit oligomerization, allowing other regions in ligand-binding (tuning) ORs to evolve novel odorant recognition abilities. In Orco–OR complexes, the specific tuning OR alters cation permeability, is affected by pharmacological channel blockade, and is involved in current rectification, suggesting that both Orco and OR subunits form the central ion-conducting pore of the channel

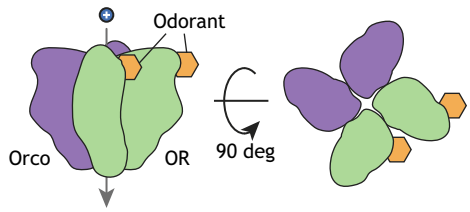

Fig. 2. Subunit contributions to channel properties of an Orco–OR complex. Heterotetrameric insect odorant receptors consist of two different subunits: (1) the obligate Orco co-receptor (purple), which is functionally conserved across insects and is necessary for OR dendritic localization; and (2) a variable ligand-binding (tuning) OR subunit responsible for odorant sensitivity (green), which affects the probability of pore opening and mediates excitatory or inhibitory responses. Note that Orco can also assemble as a homotetramer and is modulated by phosphorylation. Based on previous structure–function studies (Benton et al., 2006) and the cryo-EM structure of an Orco homomeric channel (Butterwick et al., 2018), Orco–OR complexes probably assemble with a 2:2 subunit stoichiometry; the complex forms a cation-conducting pore, influencing cation permeability. The contributions of each subunit to the overall channel properties are depicted.

(Fig. 2). Furthermore, Orco–OR complexes are likely composed of two Orco subunits and two tuning OR subunits based on split-YFP oligomerization studies (Fig. 2) (Benton et al., 2006) but this stoichiometry needs to be confirmed by future studies.

As described above, each *Or* gene is expressed in only a small fraction of ORNs, and each ORN generally expresses only one *Or* gene, with a few exceptions where two or three *Or* genes may be co-expressed in one ORN, usually when they are closely related (Martin et al., 2013). *orco* is the only highly conserved member of the *Or* gene family and is expressed in all OR-expressing ORNs, but not in GR- or IR-expressing ORNs. In *Drosophila*, *orco* is the last *Or* gene to be expressed in pupae, after all other tuning *Or* genes (Jones et al., 2005; Larsson et al., 2004), although its regulation has not been examined in detail. None of the transcription factors identified from an RNA interference (RNAi) screen for their ability to regulate *Drosophila Or* genes appear to also regulate *orco* expression (S.J. et al., unpublished observations). In *Drosophila*, Orco does not appear to play a developmental role in ORNs: *orco* mutant flies do not show defects in the morphology of ORNs and glomeruli during development (Larsson et al., 2004). In contrast, Orco displays a clear role in neural development in ants (Trible et al., 2017; Yan et al., 2017). However, localization and stabilization of ORs in the dendritic membranes and possibly correct protein folding of tuning ORs are dependent upon Orco in *Drosophila*: in *orco* mutant flies, tuning ORs are mis-localized to ORN cell bodies rather than dendrites (Benton et al., 2006; Larsson et al., 2004).

Development of the olfactory system in insects

Two essential questions in chemosensory neurobiology are: how is the wide diversity and specific expression of ORNs produced?; and how are ORNs assembled in neural circuits? The development of sensory systems in animals follows two different processes of cell fate decision: deterministic versus stochastic. In the deterministic process, differentiation of each neuron is determined by the genetic regulatory program, including signaling events and sequential expression of transcription factors that produce highly reproducible outcomes, while in the stochastic process, neuronal cell types are chosen by chance: a naive precursor cell randomly selects to express one of the multiple *Or* genes (sensory receptor expression), with particular probabilities for each gene.

Deterministic versus stochastic cell fate determination has been widely studied in the visual system in insects and vertebrates (Chen

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et al., 2012; Ebadi et al., 2018; He et al., 2012; Johnston and Desplan, 2008; Perry et al., 2016). In the olfactory system, it is generally assumed that insects utilize deterministic mechanisms for the specification of ORN classes, based on studies in *Drosophila*, while in mammals, such as mice, each ORN stochastically expresses one of a thousand *Or* genes (Magklara and Lomvardas, 2013). However, the very large number of *Or* genes (300–500) specifically expressed in ants suggests that stochastic mechanisms might be involved in the development of their ORNs as it would be difficult to control so many genes with the repertoire of transcription factors involved in ORN determination.

Development of ORNs

Adult insects have two main olfactory organs: antennae and maxillary palps. The dendrites of ORNs are located in hair-like structures called sensilla on the surfaces of these organs (Brochtrup and Hummel, 2011). There are three types of sensilla: coeloconic, basiconic and trichoid. Although these three sensilla types are highly conserved in all insects, an additional sensilla type, sensillum placodeum, has been described in other insects such as the scarab beetle *Anomala cuprea* (Leal and Mochizuki, 1993) and Japanese beetle *Popillia japonica* (Kim and Leal, 2000). Interestingly, female-specific 9-exon ORs are highly enriched in basiconic sensilla (McKenzie et al., 2016; Zhou et al., 2012) of queens and workers, suggesting that this sensillum type plays important roles in eusociality.

In *Drosophila*, ORNs in the antenna develop from multipotent sensory organ precursors (SOPs) located in the larval antennal imaginal discs. The differentiation of SOPs into individual ORNs is precisely regulated by multiple transcription factors and is divided into three steps: pre-patterning of SOPs, SOP selection and Notch-mediated neurogenesis (Fig. 3). The roles of some regulators are conserved, while those of others differ between *Drosophila* and other insects (see below).

Pre-patterning of SOPs is induced by a group of critical transcription factors including Rotund, which is involved in spatial patterning during antennal disc development (Li et al., 2015, 2013). Rotund, along with other transcription factors, such as BarH1/H2,

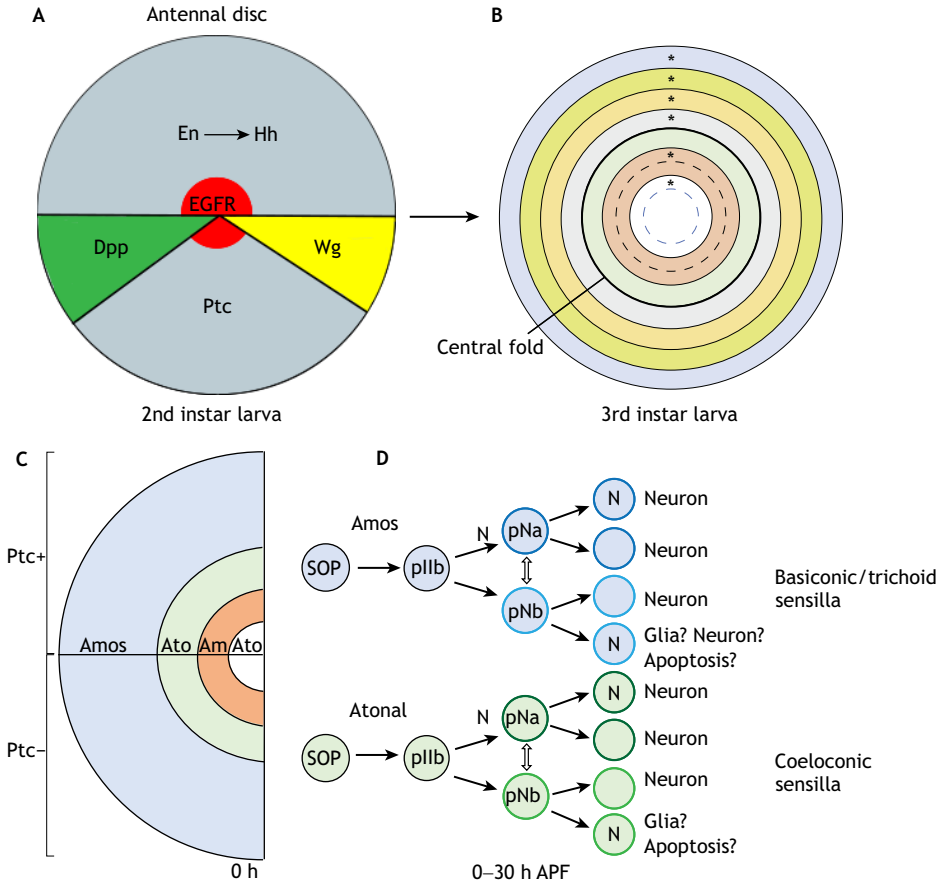

Fig. 3. Development of olfactory receptor neurons in *Drosophila*. (A) Early patterning of the antennal disc in second instar larvae: expression of Engrailed (En), Hedgehog (Hh), Wingless (Wg) and Decapentaplegic (Dpp) divides the antennal disc into different zones. Hh expression in the posterior disc is activated by En. Expression of Dpp and Wg is then initiated by Hh signals in the anterior disc. Epidermal growth factor (EGF) expression is activated where Dpp and Wg gradients meet. (B) The antennal disc in the third instar larvae is composed of seven rings of combinations and gradients of components of a gene regulatory network. Each ring gives rise to different types of sensilla. Later in the 3rd instar larvae, the two inner rings will divide further (dashed lines). Asterisks represent the rings that will continue to express Amos; rings without asterisks will express Atonal in the late third instar larvae. (C) Hedgehog-mediated Patch (Ptc) expression and the transcription factors Atonal (Ato), Amos (Am) and Lozenge (which is expressed in Amos-positive rings) specify sensory organ precursors (SOPs) which are selected at the onset of pupal formation (0 h after puparium formation, APF). (D) SOPs generate olfactory receptor neurons (ORNs) under the control of Notch (N) signaling. ORN classes are further diversified based on Notch signaling levels in each basal cell (pNa and pNb). Blue cells represent the Amos-positive lineage; green cells represent the Atonal-positive lineage. pNa cells are Notch positive, and these cells and their descendants are indicated with a darker outline. Lighter outlines indicate Notch-negative pNb cells and their descendants. The Notch-positive descendants of pNb cells can take alternative fates – usually, they undergo apoptosis, but in a few cases, they become either glia or another neuron.

Bric-à-brac, Apterous and Dachshund, pattern the antennal disc into seven concentric rings, distinct zones that specify SOPs into particular sensillum subtypes that hold different combinations of ORNs (Fig. 3) (Li et al., 2016). Interestingly, this patterning of diversification is somewhat similar to the spatial patterning of neurons in the optic lobe (Nériec and Desplan, 2016), suggesting that similar regulatory processes may give rise to completely distinct neurons based on their zonal origin.

The second step, SOP selection, is initiated by Distal-less (Dll) and its cofactor Homothorax (Hth), which in turn trigger the expression of Atonal. Atonal is both necessary and sufficient for the specification of olfactory coeloconic sensilla on the antenna and for all the olfactory basiconic sensilla on the maxillary palp. In contrast, Amos determines the development of basiconic and trichoid sensilla on the antennae. Regulation of Amos is independent from that of Atonal and relies on Lozenge (lz) to distinguish SOPs during sensillum development. Weak expression of *lz* in *Amos*-positive SOPs results in a trichoid sensilla fate while strong *lz* expression gives rise to basiconic sensilla (Fig. 3) (Goulding et al., 2000; Gupta and Rodrigues, 1997).

After the neuronal fate of SOPs is established and their differentiation potential is restricted, they undergo asymmetric cell divisions, and their cell fate is determined by either turning on or turning off the Notch signaling pathway, giving rise to the different sets of ORNs that occupy each sensillum subtype. Notch signaling plays a major role in the segregation of cell fate decisions through inter-cellular interactions (Fig. 3) (Endo et al., 2007, 2011).

As a large number of *Or* genes have been identified in Hymenoptera, including honeybees and ants, an intriguing question is: how do the developmental processes of ORNs change during the evolution of insects with highly complex social organizations? In *Drosophila*, mutation of *orco* inactivates ORNs but does not affect the neuroanatomy of ORNs and glomeruli during development (Larsson et al., 2004), although it leads to the later degeneration of axons and morphological changes in glomeruli after eclosion (Chiang et al., 2009). In contrast, *orco* mutations in two ant species, *Harpegnathos saltator* and *Oocerea biroi* (Trible et al., 2017; Yan et al., 2017), not only block ORN-mediated social communication but also dramatically reduce the number of ORNs. This is similar to ORN inactivation in mice, which led to the loss of ORNs (Yu et al., 2004). Mice utilize a completely different mechanism to determine ORN classes and choose stochastically to express one of >1400 *Or* genes (Monahan and Lomvardas, 2015), while *Drosophila* stereotypically express each of 60 *Or* genes through combinations of transcription factors. An intriguing question is, therefore, do ants, like mice, use stochastic, rather than deterministic cell fate determination, or perhaps a combination of the two?

Axon targeting and antennal lobe segregation

Insect ORNs develop during the pupal stage when they establish class-specific projections to the developing antennal lobe. In *Drosophila*, this process occurs during the first 50 h after puparium formation (APF), i.e. before *Or* genes are turned on in the antenna, suggesting that, unlike in mice, ORs do not participate in targeting to glomeruli.

Although axonal targeting of different classes of adult ORNs to their appropriate antennal lobe glomeruli is a stepwise process in insects, the order of these steps varies between species. *Drosophila* axonal targeting starts with the pre-patterning of the antennal lobe by PNs, followed by the proper contact of ORNs to their corresponding PNs (Jefferis and Hummel, 2006; Prieto-Godino et al., 2012; Rodrigues and Hummel, 2008). By 50 h APF, PN dendrites and ORN axon terminals have built a precise map of individual glomeruli that have anatomically distinct structures (Jefferis et al., 2004; Jhaveri et al., 2000; Tissot et al., 1997). In contrast, in the moth *Manduca sexta*, axons of ORNs project to the antennal lobes before the dendrites of PNs (Malun et al., 1994; Oland et al., 1996). Nevertheless, the olfactory sensory axons are critical for the development of antennal lobes: in moths and honeybees, elimination of sensory axons results in the failure of glomerular development (Hildebrand et al., 1979; Monti-Graziadei and Graziadei, 1992; Oland and Tolbert, 1987; Stout and Graziadei, 1980).

Multiple factors are involved in the regulation of the specification and dendrite targeting of PN classes as well as axon projection and targeting of ORN axons. Two POU-domain transcription factors, Acj6 and Drifter, and a zinc finger transcription factor, Jing, are expressed in exclusive groups of PNs originating from distinct neuroblasts. They are required both for dendrite targeting of PNs and for axonal targeting of ORNs (Komiyama et al., 2004, 2003; Nair et al., 2013).

Hedgehog (Hh) signaling controls axon targeting of ORNs in two steps. Based on Hh level, SOPs in the antennal disc can be divided into two subgroups, which in turn create two ORN populations with different levels of the Patched (Ptc) receptor. This Hh–Ptc positional information is critical for later axon targeting: ORN axons with low levels of Ptc only target the antennal lobe area with high brain-derived Hh (Chou et al., 2010).

Interestingly, many ORN classes express a unique combination of members of the defective proboscis extension response (Dpr) and Dpr-interacting protein (DIP) families that function as interacting partners. ORNs that target neighboring glomeruli have different combinations of Pdr/DIPs, while ORNs with very similar DIP/Dpr combinations project to distant glomeruli in the antennal lobe. Perturbation of DIP/Dpr genes does not affect ORN–PN matching, but leads to local projection defects in which perturbed axons invade incorrect glomeruli (Barish et al., 2018).

Although mechanisms of axon targeting and glomeruli formation have been extensively analyzed in *Drosophila*, studies in other insects have identified different regulators. In *Manduca sexta*, the transmembrane form of Fasciclin II (a homolog of vertebrate neural cell adhesion molecule, NCAM) is found on a subset of ORN axons and is important for glomerulus formation. Different isoforms of Fasciclin II are expressed by a subset of developing ORNs and by olfactory nerve glial cells during formation of glomeruli in *Manduca* (Higgins et al., 2002). Epidermal growth factor receptor (EGFR) is also found on ORN axons, and mediates ORN axon sorting and extension in the developing olfactory system. Blocking EGFR caused ORN axon stalling and loss of axon fasciculation (Gibson and Tolbert, 2006).

Different mechanisms may be utilized for axon targeting and formation of glomeruli in different species. For example, the Orco-OR complex plays distinct roles in glomerulus formation in *Drosophila* versus ants. Mutations in *orco* do not affect the development of glomeruli in *Drosophila* (Chiang et al., 2009; Larsson et al., 2004), while in *orco* mutant worker ants, only ∼20% of glomeruli remain in these two ant species that normally contain 250–500 glomeruli, suggesting that the function of Orco in regulating neural development is conserved in ants, and perhaps in all hymenopteran eusocial and solitary insects and in coleopteran flour beetles, where *Or* genes have undergone dramatic expansion (Trible et al., 2017; Yan et al., 2017). Although severely reduced, there are still 62 glomeruli remaining in *Harpegnathos* mutant female ants, more than the number of ORN subtypes that express

other chemosensory receptors, i.e. GR- and IR-expressing ORNs (Yan et al., 2017). This suggests that a small number of OR-expressing ORNs still survive without Orco. Dependence on Orco for ORN development has also been found in *Harpegnathos* male ants, which normally contain less than a third the number of glomeruli compared with females. Further investigation is required to identify ORN classes that display differential reliance on Orco for their development/survival.

However, the regulatory mechanisms that direct ORN axons to their correct glomeruli have not been investigated in ants and other insects. On the one hand, ants may have similar mechanisms to *Drosophila* for precise deterministic axon projection. On the other hand, OR or ORN activity may be involved in the regulation of axon targeting, as in mice. Furthermore, it is established in mice that the presence of a single OR protein type in an ORN triggers negative feedback suppression of the expression of other *Or* genes (Serizawa et al., 2003), a mechanism that has not been found in insects. Do some insects, like ants, utilize different mechanisms than those of *Drosophila* for ORN development, axon targeting and antennal lobe glomeruli segregation as well as *Or* expression? These questions deserve further investigation.

What happens to PNs in *orco* mutant ants that have lost most of their glomeruli? The size of the remaining glomeruli in mutant female ants is increased compared with wild-type (Yan et al., 2017). This is likely the result of the absence of ORN axon projection, which might lead to the failure to split pre-glomeruli into mature glomeruli. This hypothesis is consistent with the development of glomeruli in *Drosophila*: the formation of pre-glomeruli depends on PNs, rather than ORNs, and ORNs play a role in fine-tuning the final shape and size of glomeruli. However, this order is reversed in *Manduca* where ORNs likely form pre-glomeruli and PNs shape them into mature glomeruli (Malun et al., 1994; Oland et al., 1996). Therefore, it is important to address the temporal pattern of glomerulus formation in ants and in other insects.

Most insects follow the 'one neuron, one receptor' rule, in line with the evidence that the number of *Or* genes roughly equals the number of glomeruli. However, the arrangement of glomeruli in locusts is different (Ignell et al., 2001); although locusts contain ∼120 *Or* genes (Li et al., 2018), each ORN (and PN) innervates several of the approximately 1000 glomeruli (Anton and Hansson, 1996; Ernst et al., 1977).

Expression of *Or* genes

The final step of ORN differentiation is *Or* gene choice, which has mainly been investigated in *Drosophila*. The *Drosophila* OR family consists of 62 receptor proteins transcribed from 60 *Or* genes (two *Or* genes give rise to four proteins with alternative splicing). *Or* genes are dispersed on the three major chromosomes, while a few exhibiting high homology are found in small clusters, suggesting recent gene duplication. Typically, each ORN expresses a single *Or* gene, with a few exceptions: the clustered *Or* genes are mostly co-expressed in the same ORNs (Jones et al., 2005; Robertson et al., 2003).

The first *Or* genes start to be expressed at about 40 h APF, after the development of ORNs and diversification of sensillum subtypes, as described above. Two DNA regulatory elements, dyad-1 and Oligo-1, are present at unexpectedly high frequencies upstream of maxillary palp *Or* genes, but not of antennal *Or* genes. Disruption of dyad-1 in the promoter of *Or* genes abolishes expression. Elimination of Oligo-1 results in ectopic expression in the antenna, suggesting that Oligo-1 restricts *Or* expression to the maxillary palp (Ray et al., 2007).

In the maxillary palp, lz and Acj6 regulate the expression of *Or* genes that can be divided into three classes: those that require both Acj6 and lz, Acj6 alone, or lz alone (Ray et al., 2007).

Interestingly, Acj6 has 13 alternatively spliced forms and individual splice forms can either activate or repress different sets of *Or* genes (Bai and Carlson, 2010). Another POU domain factor, Pdm3, also regulates *Or* expression as mutation of *pdm3* severely affects the expression of Or42a in the maxillary palp and Or59b in the antennae (Jafari and Alenius, 2015; Tichy et al., 2008).

An RNAi screen in post-mitotic ORNs identified seven transcription factors as regulators of the maintenance of OR expression in the adult olfactory system: Acj6, Fer1, Xbp1, E93, Onecut, Sim and Zf30c (Jafari et al., 2012). Each of these transcription factors is required to maintain the expression of subsets of *Or* genes in the adult ORNs. Fer1, Xbp1, E93, Onecut, Sim and Zf30c belong to different protein families and have overlapping expression in the antenna. Binding motifs for Onecut, Acj6, Xbp1 and Fer1 are found upstream of a large number of *Or* genes. In some cases, these binding motifs are required for *Or* expression, while in other cases, the motifs are necessary for preventing *Or* genes from being expressed in other ORN classes (Jafari and Alenius, 2015; Jafari et al., 2012). In addition, chromatin modulators, such as Atrophin, Alhambra and Su(var)3-9, assist these transcription factors in restricting *Or* expression to specific sensillum subtypes in different environmental conditions (Alkhori et al., 2014; Hueston et al., 2016; Jafari and Alenius, 2015; Sim et al., 2012). Furthermore, an RNAi screen for genes affecting the expression of Or67d in a single ORN class has demonstrated that 35 different transcription factors, chromatin regulators and embryonic patterning genes are required for *Or67d* expression. Out of these, 12 are required in the final stage of ORN specification (Chai et al., 2019).

Function and regulation of the olfactory system in insects

Odorant perception is a complex process that has evolved to sense, identify, evaluate and memorize the vast number of odors in the environment. General odorants and pheromones induce behavioral responses through ORs in insects (Hansson and Stensmyr, 2011). Some ORs respond to a wide range of odorants (broadly tuned) while others only respond to very few odorants (narrowly tuned).

Function of ORNs

How does the development of ORNs and assembly of neural circuits shape the functions of the olfactory system? In *Drosophila*, initial odor discrimination is performed by different types of chemosensory sensilla. Generally, ORNs responding to pheromones are located in trichoid sensilla; food odors are detected by ORNs in basiconic sensilla; and acids and amines are sensed by ORNs in coeloconic sensilla (Benton et al., 2009; Hallem and Carlson, 2006; Kurtovic et al., 2007). There are exceptions: in ants, CHC pheromones are mainly detected by ORNs in basiconic sensilla (Ozaki et al., 2005; Sharma et al., 2015).

The second level of odorant perception is the binding of odorants to receptors. The three main types of chemosensory receptors that are expressed in ORNs allow insects to discriminate between major groups of volatile odorants. ORs are activated mostly by acetates, aldehydes and aromatics, IRs mainly respond to ketones, acids and amines, and a pair of GRs, Gr21a and Gr63a, detects CO_2 (Hallem and Carlson, 2004; Hallem et al., 2006; Jones et al., 2007; Kwon et al., 2007; Silbering et al., 2011). OR proteins are trafficked to ORN dendrites, which are embedded in the sensilla, to detect and evaluate airborne chemical cues. The first detailed analysis of *Drosophila* OR function came from the odorant response profiles of OR receptors that were tested using single unit electrophysiological analysis. These recordings were obtained either by ectopic expression of ORs in an 'empty neuron' (a neuron in which the

endogenous *Or* has been mutated) or by direct expression in native ORNs (Dobritsa et al., 2003; Hallem and Carlson, 2006; Hallem et al., 2004). In the first experiments, 24 ORs were tested against a panel of 109 chemically diverse odorants. The ORs showed very different profiles, from narrowly to broadly tuned, representing both specialist and generalist characteristics. The broad range of different odorants that could be detected by each OR, some of which overlapped between different ORs, indicates that odorant sensing in *Drosophila* follows a combinatorial code: each OR is able to respond to multiple ligands, and a single ligand can activate multiple ORs and consequently multiple glomeruli in the antennal lobe (Clyne et al., 1997; de Bruyne et al., 2001; Hallem and Carlson, 2006; Hallem et al., 2004; Malnic et al., 1999).

High doses of odorants may trigger non-specific binding to ORs. In order to conclude that a receptor or ORN is broadly tuned, the actual amount of stimulus that reaches the sensory neuron is crucial, especially if the active compounds are structurally unrelated and have different volatilities or solubilities. Recent studies suggest that many ORs are narrowly tuned and selectively activated by a few structurally relevant odorants at biologically relevant concentrations. For instance, Or22a/b was first found to respond to the three key ligands methyl hexanoate, ethyl hexanoate and ethyl butyrate with similar sensitivity at a dilution of 1:100 (Dobritsa et al., 2003; Hallem et al., 2004; Stensmyr et al., 2003). However, when reducing the concentration of odorants to 1:10,000 dilution, Or22a/b neurons showed the highest sensitivity to ethyl and methyl hexanoate (Andersson et al., 2012; Hallem and Carlson, 2006; Pelz et al., 2006). It is likely that the insect olfactory system contains both narrowly (specialists) and broadly tuned receptors (generalists), with both specifically dedicated receptors for certain compounds and combinatorial coding for others (Andersson et al., 2015; Touhara and Vosshall, 2009).

Drosophila empty neurons, along with other ectopic expression systems, such as cell culture and *Xenopus* oocytes, have been used to identify (i.e. deorphanize) the ligands of ORs from other insects (Fleischer et al., 2018), including mosquitos, silkmoths, honeybees and ants (Carey et al., 2010; Nakagawa et al., 2005; Pask et al., 2017; Slone et al., 2017; Wanner et al., 2007b). Both broadly and narrowly tuned ORs have been identified. As described above, the concentration of odorants used in the analysis is a common concern that may be confusing for the identification of broadly versus narrowly tuned ORs. Lower concentrations are likely to represent the environmental level of odorants that insects encounter. Additionally, many of the heterologous expression systems lack the odorant binding proteins and odorant degrading enzymes that typically surround native ORNs (Bohbot and Pitts, 2015; Leal, 2013). Nevertheless, the deorphanized ORs can be further analyzed *in vivo* using CRISPR-mediated mutagenesis. For example, in *Helicoverpa*, OR16 is the only OR tuned to a female-produced sex pheromone antagonist to prevent mating of males with immature females (Liu et al., 2013). Knockout of the *OR16* gene leads to abnormal mating behavior (Chang et al., 2017).

Some specialized ORs are dedicated to sensing toxic odors. For instance, Or56a in *Drosophila melanogaster* detects geosmin, a microbial odorant, and alerts flies to the presence of harmful microbes. Activation of the Or56a neuron by geosmin elicits activity in a single glomerulus (DA2), which is necessary and sufficient for avoidance. Geosmin-specific ORN homologs were identified in seven other *Drosophila* species, indicating the evolutionary conservation of geosmin detection (Stensmyr et al., 2012).

Most insects have CO_2-sensitive systems with a wide variety of structure and function (Kleineidam and Tautz, 1996; Stange and Stowe, 1999). The relevance of CO_2 also differs among species. In blood-sucking insects such as mosquitoes, CO_2 is the cue for finding the host (Dekker et al., 2002; Grant et al., 1995). CO_2 might be a component of the attractive flower odor for nocturnal moths, as flowers release considerable amounts of metabolic CO_2 (Guerenstein et al., 2004; Raguso, 2004; Thom et al., 2004). Insects such as social insects (ants, bees and termites) monitor CO_2 in their hives and control its concentration (Stange and Stowe, 1999; Weidenmüller et al., 2002).

CO_2 may serve as an attractant or as a repellent for *Drosophila*. CO_2 elicits avoidance behavior by activating Gr21a-expressing ORNs at levels as low as 0.1%. When in an active state associated with foraging, *Drosophila* might be attracted to CO_2 via another receptor, IR25a. Interestingly, Gr21a is not involved in this attraction (van Breugel et al., 2018). However, CO_2 is detected by two GR-expressing ORNs (Gr21a and Gr63a) as an attractive odorant in mosquitos (Jones et al., 2007), suggesting that the same odorant may induce different, or even opposite behavioral responses in different species, probably due to the different neural circuits in the higher-order neurons. Indeed, males in two *Drosophila* species, *D. melanogaster* and *D. simulans*, differentially respond to 7,11-heptacosadiene, produced by female *D. melanogaster* as a sex pheromone. The pheromone induces homologous Pickpocket PPK23 chemosensory neurons, but this signal is differentially propagated to the P1 neurons in the central circuits. As a result, it promotes courtship in *D. melanogaster* males but suppresses courtship in *D. simulans* males (Seeholzer et al., 2018).

A few ORs mediate odorant-induced oviposition. A dedicated OR in *Drosophila*, Or19a, is activated by citrus volatiles. Activation of the neurons expressing Or19a is necessary and sufficient for the selective oviposition on citrus fruits. Similarly, ethylphenol activates Or71a neurons, leading to increased feeding and oviposition (Dweck et al., 2015a).

A subset of narrowly tuned ORs respond to pheromone components and induce different behaviors. For example, cis-vaccenyl acetate (cVA) produced by male flies induces courtship behavior, possibly via Or67d and Or65a neurons, while Or88a and Or47b neurons mediate the response to fatty acid methyl esters and induce copulation and attraction, but not courtship behavior (Dweck et al., 2015b; Ejima et al., 2007; Pitts et al., 2016). In addition, 9-tricosene is detected via OR7a. This pheromone is deposited by males and acts as an aggregation pheromone and as an oviposition guidance cue for females (Lin et al., 2015).

The best-studied insect pheromone is bombykol, which is released from sexually mature female silkmoths and activates male reproductive behavior. The antennae of male silkmoths contain bombykol-specific pheromone receptor neurons that express BmOR-1, which is male specific (Nakagawa et al., 2005; Sakurai et al., 2004). Female silkmoth antennae also show biased expression of BmOR-19 and BmOR-30, the ligands of which are unknown (Wanner et al., 2007a). These aspects of OR expression and function in insects suggest that ORs are biologically responsible for the recognition of relevant odorants and pheromones from the chemical environment.

Single sensillum recording has been extensively used to analyze electrophysiological responses to odorants. Whether single sensillum recording can provide resolution at the neuronal level depends on the complexity of the sensilla. *Drosophila* sensilla only contain one to four ORNs, and it is therefore easy to distinguish the electrophysiological signal in each ORN. In contrast, up to 130 ORNs are found in each sensillum in the ant *Camponotus japonicus* (Nakanishi et al., 2009). Recent analysis on *Harpegnathos* ants

demonstrates that the reproductive pseudo-queens, compared with workers, display overall reduced responses to CHCs, including the queen pheromone (Ghaninia et al., 2017). Although the study could not analyze responses at the individual neuronal level, it provides an explanation of the differential responses to the queen pheromone between castes.

As described above, the 9-exon family constitutes more than 30% of all *Or* genes (118 out of 347 in *Harpegnathos*) (Zhou et al., 2012). In addition, 9-exon *Or* genes are mainly expressed in ORNs located in female (worker)-specific basiconic sensilla, suggesting that they are involved in mediating CHC pheromone perception, as males do not respond significantly to pheromone stimuli. Heterologous expression of a number of 9-exon ORs from *Harpegnathos* showed that they function as highly specific CHC detectors (Pask et al., 2017). However, ant ORs from outside the 9-exon family also respond to odorants and pheromones (Slone et al., 2017), suggesting that combinations of functionally similar ORs may facilitate the ability of ants to detect a large variety of social cues and discriminate castes, nestmates versus non-nestmates, etc., based on the subtle differences of their pheromones (Carlin and Hölldobler, 1983; Ozaki et al., 2005).

Regulation of ORN responses

The responses of the ORNs can be regulated by several external and internal factors. One proposed regulatory mechanism is OR adaptation to strong and continuous odorant presence: a significant decrease in spike amplitude can be seen in single neuron recordings of ORNs during continuous odor stimulation. The adaptation of Orco–OR receptors appears to be mediated by odorant-induced dephosphorylation of serine 289 of Orco and desensitization of ORNs (Guo et al., 2017).

Another form of selective inhibition is triggered by mixtures of odors. A mixture of attractant and repellent odors elicits strong inhibition in ORNs which are otherwise attractant (Mohamed et al., 2019). Repulsive odors can also cause inhibition of basal spike firing in olfactory sensory neurons. Such a bidirectional code with both odor-evoked inhibition and odor-evoked excitation in a single ORN increases the odor-coding capacity, providing more efficient sensory coding (Cao et al., 2017).

In *Drosophila*, the dendrites of up to four types of ORNs interact within a single sensillum, which represents another layer of regulation. These neurons can influence their neighbors' response to odors at the dendritic level. This interaction of ORNs within a shared space allows the insect a more precise orientation along a gradient by increasing the contrast between different odorants. For instance, upon stimulation of the ab3 sensilla, which houses two ORNs expressing Or22a (ab3A) or Or85b (ab3B), with a prolonged dose of methyl hexanoate, the ab3A neuron (Or22a) maintains a train of action potentials. In this background, a pulse of 2-heptanone causes the ab3B (Or85b) neuron to fire concomitantly, with a concomitant marked reduction in the firing of ab3A (Su et al., 2012).

In the mosquito *Anopheles gambiae*, the gene encoding a subunit of the CO_2 receptor, AgGr22, is significantly up-regulated in host-seeking females, consistent with a significant increase in sensitivity of CO_2-responsive neurons (cpA). Female *A. gambiae* respond to CO_2 regardless of their maturation, but the onset of host seeking enhances their sensitivity and speed of activation at relevant doses of CO_2, which is detected by the maxillary palps (Omondi et al., 2015).

Environmental factors, such as temperature and nutrition, may also modify antennal electrical responses of ORNs and change behavior. Microarray transcriptomic studies of the third antennal segments of high-temperature-acclimated flies demonstrated changes in the expression of nine members of the OR family: four displayed overexpression and the other four plus Orco showed downregulation. Indeed, low temperature and starvation have been also connected to alteration of some ORs (Jafari and Alenius, 2015; Riveron et al., 2009).

Conclusion

Great progress has been made in understanding the evolution of ORs, as well as the development and function of ORNs in insects. As a result, we now have a much better understanding of how insects sense and interpret environmental chemical cues, ultimately impacting their behavior. Further investigations, including functional analysis using genetic tools, should be performed both in *Drosophila* and in other insect species, to understand general principles of neural development, e.g. stochastic versus deterministic cell fate decision, as well as the molecular and cellular mechanisms that lead to the diversity and function of neural systems in more than one million insect species.

Competing interests

The authors declare no competing or financial interests.

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