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PLASTICITY IN THE SENSORY SYSTEMS OF INVERTEBRATES

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Elzbieta M. Pyza

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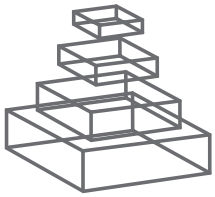

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PLASTICITY IN THE SENSORY SYSTEMS OF INVERTEBRATES

Topic Editor:
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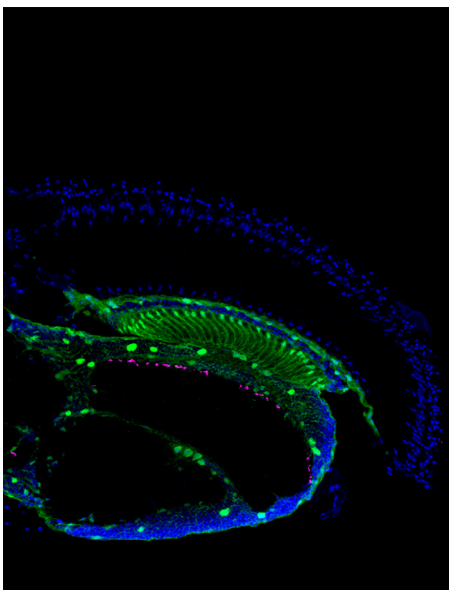

Confocal image of the visual system of *Drosophila melanogaster*. The first visual neuropil (lamina) of the optic lobe is a site of light-dependent, activity-dependent and circadian plasticity of synapses, neurons and glial cells (Pyza, 2010; Gorska-Andrzejak, 2013). Blue – cell nuclei labelled with DAPI, green – glial cells, magenta – terminals of clock cells immunoreactive to pigment-dispersing factor (PDF), one of neurotransmitters in the circadian system.
(photo by Jolanta Górska-Andrzejak)

Pyza E. (2010). Circadian rhythms in the fly's visual system. In: Darlene A. Dartt, ed. Encyclopedia of the Eye, Vol 1. Oxford: Academic Press, pp.302-311.
Górska-Andrzejak J. (2013). Glia-related circadian plasticity in the visual system of Diptera. Front. Physiol. 4:36. doi 10.3389/fphys.2013.00036.

The visual, olfactory, auditory and gustatory systems of invertebrates are often used as models to study the transduction, transmission and processing of information in nervous systems, and in recent years have also provided powerful models of neural plasticity.

This Research Topic presents current views on plasticity and its mechanisms in invertebrate sensory systems at the cellular, molecular and network levels, approached from both physiological and morphological perspectives. Plasticity in sensory systems can be activity-dependent, or occur in response to changes in the environment, or to endogenous stimuli. Plastic changes have been reported in receptor neurons, but are also known in other cell types, including glial cells and sensory interneurons. Also reported are dynamic changes among neuronal circuits involved in transmitting sensory stimuli and in reorganizing of synaptic contacts within a particular sensory system. Plastic changes within sensory systems in invertebrates can also be reported during development, after injury and after short or long-term stimulation.

All these changes occur against an historical backdrop which viewed invertebrate nervous systems as largely hard-wired, and lacking in susceptibility especially to activity-dependent changes. This Research Topic examines how far we have moved from this simple view of simple brains, to the realization that invertebrate sensory systems exhibit all the diversity of plastic changes seen in vertebrate brains, but among neurons in which such changes can be evaluated at single-cell level.

Table of Contents

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EDITORIAL
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Plasticity in invertebrate sensory systems

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Keywords: insects, *C. elegans*, lesion-induced plasticity, experience-induced plasticity, circadian plasticity

The Research Topic presented in this issue of Frontiers in Invertebrate Physiology is on Plasticity in Invertebrate Sensory Systems and comprises a total of eight articles. These cover various aspects of sensory plasticity observed not only at the level of neurons but also in behavioral adaptations that result from plastic changes in the nervous system. Neuronal plasticity has been reported in both vertebrate and invertebrate nervous systems and has mostly been documented during development. The phenomenon of plasticity also occurs in the adult nervous system, however, after injury—lesion-induced plasticity—and also after stimulation—experience-induced plasticity. Moreover, plasticity can be a process reflecting rhythmic changes in the environment, either during the day and night or in the seasons throughout the year. This type of plasticity is driven by rhythmic changes in the external environment—daily plasticity—and/or it may be generated endogenously, by circadian clocks—circadian plasticity. In invertebrates, neuronal plasticity has been reported mainly in molluscs and insects as various responses involving axon sprouting and synapse formation.

The term plasticity has been used in neuroscience for over a century and many scientists have applied this term to any change in the brain. Nowadays physiological changes at synapses after stimulation, originally in the form of long-term potentiation (LTP), and modifications in synaptic transmission as a result of learning, are regarded as fundamental plastic processes in the nervous system. However, a wide range of evidence has accumulated so far that the term plasticity can also be used to give an account of temporary or permanent structural changes of synapses, neurons and glial cells in response to internal and external stimuli.

The first examples of these phenomena that have been observed occur during development and after emergence, during the so-called critical period of increased sensitivity, and were reported as changes in the brain's final wiring that occur in response to early sensory experience. Later, changes in brain wiring were reported to occur not only during development and in early life but also in the mature brain. Now it is commonly accepted that the mature brain is plastic and that the extent of neuroplasticity is one of the brain's most amazing features. It allows an organism to adapt to new environments and to learn even until the end of life. Plasticity of the brain does, however, decrease with age, strongest changes occurring in young animals, especially during a period of enhanced activity dependence, the critical period. Although plasticity may occur in various regions of the nervous system the most striking changes have been observed in sensory systems and in the centers for learning and memory in the brain.

As shown in the articles published within this Topic Issue the nematode worm *Caenorhabditis elegans* and various insect species are good models to study neuroplasticity and its mechanisms. Despite their often being considered hard-wired, the nervous systems of invertebrates are in fact plastic, just as in vertebrates, both during development and in the adult. After injury, as reported in the article by Pfister et al. (2012) neurite outgrowth occurs. For example deafferentated neurons in the auditory system of orthopteran insects undergo dendritic and axonal growth. This leads to a gain of function, but the most surprising result observed by the authors is a dimorphic regeneration in response to this type of injury. Lesion-dependent neuroplasticity of the peripheral auditory nerve is also indicated in another article, by Lakes-Harlan (2013) who reports that the lesioned nerve regrows and forms new synaptic contacts. In addition there are also changes in the central nervous system in which sprouting of axon collaterals occurs. The article by Pflüger and Wolf (2013) also gives an example of plasticity in Orthoptera. In locusts, these authors observe activity-dependent plasticity in another sensory system, in wind-sensitive hair receptors of the sensorimotor system.

Several articles within the Topic Issue focus on experience-dependent plasticity, and various forms of learning during development and also in adults. The article by Bozorgmehr et al. (2013) details the influence of experience on habituation, the simplest example of learning in *C. elegans*, while the article by Arenas et al. (2013) reports plasticity induced by social experience in the honeybee. In both cases, *C. elegans* expressing a simple behavior and the more complex individual and social behaviors of the honeybee, structural and functional changes occur in various sensory systems and these affect learning and behavior. In turn Dylla et al. (2013) report a form of associative learning called trace conditioning.

Finally two articles provide examples of daily and circadian plasticity in insects. In flies, neurons and glial cells undergo size changes during the day and night and because this cyclical plasticity is also maintained under conditions of constant darkness it must be endogenous, generated by a circadian clock (Górska-Andrzejak, 2013). Moreover this author gives convincing examples that glial cells are important for plasticity of the neurons they surround. In turn clock neurons, which generate circadian rhythms observed in behavior and other processes, including cyclical structural changes in neurons and glial cells of the visual system in flies, are under pressure of environmental stimuli (Shiga, 2013). Their morphological changes depend on

photoperiod. In long days they have longer commissural fibers. The articles published with this Topic Issue show that many factors affect the structure and physiology of neurons in invertebrates, no less than in the brains of vertebrate species. Thus, the nervous systems of invertebrates prove themselves to be not only plastic in response to injuries, so as to re-establish damaged connections and functions, but are also remodeled in response to external and internal signals.

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Mechanisms of plasticity in a *Caenorhabditis elegans* mechanosensory circuit

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Despite having a small nervous system (302 neurons) and relatively short lifespan (14–21 days), the nematode *Caenorhabditis elegans* has a substantial ability to change its behavior in response to experience. The behavior discussed here is the tap withdrawal response, whereby the worm crawls backwards a brief distance in response to a non-localized mechanosensory stimulus from a tap to the side of the Petri plate within which it lives. The neural circuit that underlies this behavior is primarily made up of five sensory neurons and four pairs of interneurons. In this review we describe two classes of mechanosensory plasticity: adult learning and memory and experience dependent changes during development. As worms develop through young adult and adult stages there is a shift toward deeper habituation of response probability that is likely the result of changes in sensitivity to stimulus intensity. Adult worms show short- intermediate- and long-term habituation as well as context dependent habituation. Short-term habituation requires glutamate signaling and auto-phosphorylation of voltage-dependent potassium channels and is modulated by dopamine signaling in the mechanosensory neurons. Long-term memory (LTM) for habituation is mediated by down-regulation of expression of an AMPA-type glutamate receptor subunit. Intermediate memory involves an increase in release of an inhibitory neuropeptide. Depriving larval worms of mechanosensory stimulation early in development leads to fewer synaptic vesicles in the mechanosensory neurons and lower levels of an AMPA-type glutamate receptor subunit in the interneurons. Overall, the mechanosensory system of *C. elegans* shows a great deal of experience dependent plasticity both during development and as an adult. The simplest form of learning, habituation, is not so simple and is mediated and/or modulated by a number of different processes, some of which we are beginning to understand.

Keywords: tap-withdrawal response, *C. elegans*, non-associative learning, habituation, short-term memory, long-term memory, context conditioning

Caenorhabditis elegans (*Caeno*, recent; *rhabditis*, rod; *elegans*, nice) is a 1 mm, free-living nematode which was introduced by Sydney Brenner in 1963 as a powerful model organism. During the last 50 years scientists have taken advantage of this tiny creature to reveal functions of genes in developmental and cellular biology. Some of the characteristics which made *C. elegans* an amenable model for doing this research are its small size, short life span, and mode of reproduction. The lineage of the worm's 959 somatic cells was traced through its transparent cuticle, allowing for determination of cell fate (Sulston et al., 1983). Furthermore, *C. elegans* has a sequenced genome comprising approximately 19,000 genes, over 5000 of which have homologues in humans. All of these features contribute to the power of *C. elegans* as a valuable model for understanding molecular mechanisms of cellular plasticity in more complex creatures.

Of the 959 cells in the hermaphrodite worm, 302 are neurons. The wiring and connectivity of the *C. elegans* nervous system has been described (White et al., 1986) and can be divided into a pharyngeal nervous system containing 20 neurons and a somatic nervous system containing 282 neurons. The somatic nervous system contains about 6393 chemical synapses, 890 gap junctions, and 1410 neuromuscular junctions (Varshney et al., 2011). *C. elegans* nervous system is well-adapted to respond to a variety of sensory modalities, including mechanosensation, thermosensation, and chemosensation to mediate behavior (Giles et al., 2006). In 1990, Rankin, Beck, and Chiba were the first to report learning and memory in *C. elegans*. They found that these animals are capable of learning in the form of both short- and long-term habituation. Habituation is defined as a gradual decrease in response to repeated stimuli, which is not explained by sensory adaptation/sensory fatigue or motor fatigue (Thompson and Spencer, 1966). This review focuses on plasticity of the mechanosensory system through the life span of *C. elegans*.

MECHANOSENSORY CIRCUITS

C. elegans has a variety of sensory neurons that respond to mechanical stimuli. Activity of the touch neurons, proprioceptors, and nociceptors are modulated by mechanical force. Two protein superfamilies' are essential for transforming

mechanical stimuli into electrical signals by changing the ionic current in mechanosensory neurons: the TRP channels, and the DEG/ENaC channels. TRP channels are non-specific cation channels composed of six transmembrane alpha helix subunits, while DEG/ENaC channels are predominantly permeable to sodium and in some cases to calcium and consist of two transmembrane alpha helices. The mechanism of mechanotransduction has been broadly studied in *C. elegans* and over 10% of the neurons in the adult hermaphrodite are sensitive to external touch stimuli (Chatzigeorgiou and Schafer, 2011). The best studied of the mechanosensory circuits are the head and tail touch circuits: when a worm is touched lightly on the head it crawls backwards away from the stimulus; when a worm is touched lightly on the tail it crawls forwards away from the stimulus. The touch cell anatomical wiring diagram was determined by serial section electron micrographs (Sulston et al., 1980; White et al., 1986). Subsequently, Chalfie et al. (1985) used laser ablation of neurons to determine the function of cells in the head and tail touch circuits. By killing cells and assaying touch responses Chalfie et al. showed that response to gentle touch to the body was mediated by three mechanosensory neurons in the head (ALML, ALMR, and AVM) and by two mechanosensory neurons in the tail PLML and PLMR. In addition four pairs of interneurons were implicated: AVD, PVC, AVA, and AVB, which are often called command interneurons and integrate information onto motor neurons. Laser ablation showed that AVD and AVA are required for moving backwards to anterior touch, while PVC and AVB are involved in moving forward to posterior touch. The five touch-receptor neurons [ALM (L/R), PLM (L/R), AVM] have a very simple structure. Each cell has a single long process that ends in a synaptic branch. These neurons make synapses at synaptic branches, and along their processes (Chalfie et al., 1985). At the time of hatching ALML and ALMR are located on the left and right side, respectively, in the anterior half of the worm while PLML and PLMR are on the left and right in the posterior. AVM develops post-embryonically and is located in a ventral position (Chalfie and Sulston, 1981). In addition to light touch, the same five mechanosensory neurons respond to a mechanical stimulus delivered to the side of the agar-filled petri plate holding the worm. This vibrational stimulus is called a tap (Rankin et al., 1990) and is thought to simultaneously activate the head and tail touch circuits. In response to tap worms crawl backwards for a short distance. Through laser ablation Wicks and Rankin (1995) confirmed that the five sensory neurons and four pairs of interneurons described in Chalfie et al. (1985) were also critical for the tap withdrawal response. Wicks and Rankin (1995) also hypothesized that DVA and PVD neurons play a role in integrating the sensory input coming from the head and tail (**Figure 1**). The head and tail input triggers movement in opposite directions; the direction of movement in response to tap is thought to be dependent on imbalanced activity between forward (two sensory neurons) and backward (three neurons) sub-circuits (Chalfie et al., 1985; Wicks and Rankin, 1995). Chiba and Rankin (1990) showed that, before the post-embryonic AVM neuron develops, the circuit is balanced with two sensory neurons for each head and tail, and the behavior consists of 50% forward and 50% backward locomotion in response to tap. When AVM connects to the circuit the bias shifts to reversals (>85% of responses).

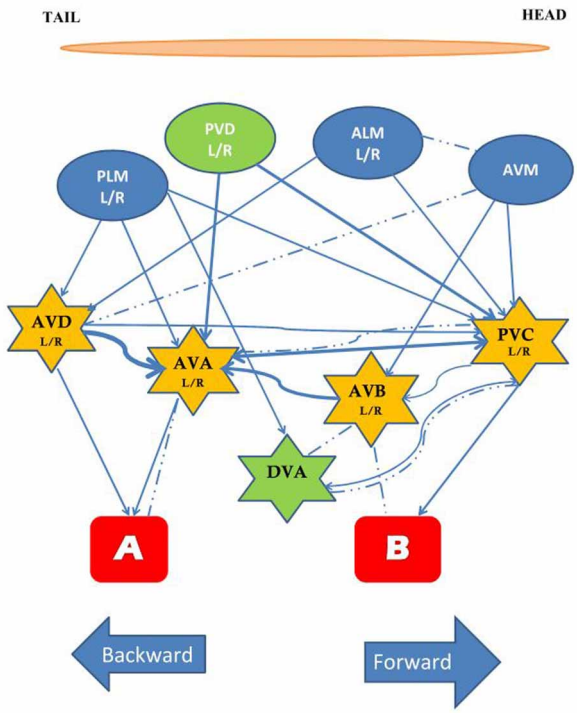

FIGURE 1 | The circuit mediating the response to tap. The touch cells are represented by ovals (blue), the interneurons by stars (yellow), and the motor neurons by rectangles (red). All neurons are bilaterally symmetrical, except AVM and DVA. The neurons in green, PVD and DVA, contribute to both forward and backward movement and may play a role in integrating the two responses. Arrows and dashed lines denote chemical and electrical connections, respectively, thickness of lines reflects relative number of synapses [Based on data from Chalfie et al. (1985), Wicks and Rankin (1995)].

ADULT LEARNING AND MEMORY: TAP HABITUATION

Sensory plasticity plays a critical role in an organisms' ability to regulate processes of attention. Animals are constantly bombarded by sensory information and do not have the attentional resources to attend to all of the inputs at the same time. In response to this, sensory systems have developed the ability to filter out stimuli that are unimportant in that they do not signal appetitive or aversive stimuli. This form of plasticity is the non-associative form of learning called habituation. Habituation is a decrease in responding after repeated presentation of a stimulus, and is distinguished from sensory adaptation and motor fatigue by the ability of a novel or noxious stimulus to rapidly return the response to original levels through dishabituation (only time will allow recovery from adaptation or fatigue). The behavioral characteristics are similar in all organisms that have been studied (Groves and Thompson, 1970). Despite the large number of studies of habituation across a broad range of organisms remarkably little is understood about the mechanisms of this form of learning. *C. elegans* offers a small tractable nervous system and

sequenced genome that might facilitate understanding the cellular mechanisms of this "simplest" form of learning. In 1990, Rankin et al. found that repeated taps administered to the side of Petri plates in which worms were cultivated resulted in habituation. They measured reversal distance after each mechanical stimulus and showed that distance decremented after repeating the stimulus 40 times at interstimulus intervals (ISIs) of 10 s or 60 s. This response decrement recovered to the baseline after a few minutes. In these studies, reversal distance and reversal probability were combined into a single measure of response magnitude by assigning worms that did not reverse to a tap a distance score of "0." To confirm this decrease in reversal magnitude as habituation they needed to rule out sensory adaptation or fatigue by showing dishabituation. To do this they applied electric shock to the agar on which worms were grown. After electric shock a tap resulted in increased reversal distance, indicating that the gradual response decrement to mechanical stimuli could be definitively called habituation (Rankin et al., 1990).

To investigate the locus of short-term habituation Wicks and Rankin (1997) took advantage of the fact that the tap withdrawal circuit significantly overlaps with the thermal avoidance and spontaneous reversal circuits at the level of the command interneurons. They tested whether habituating the tap-withdrawal response had any effect on spontaneous reversals or thermal avoidance responses. Their hypothesis was that if tap habituation training resulted in a decrement of other reversal behaviors, the site of plasticity must be localized to the common circuitry; but if habituation to tap had no effect, the site of plasticity must be at loci unique to the tap-withdrawal response. The result of their experiment showed that tap habituation training had no effect on the frequency or magnitude of spontaneous reversals or on the magnitude of reversals elicited by a thermal stimulus. Based on these results they concluded that the most likely sites of plasticity for tap habituation were the synapses between the mechanosensory neurons and the interneurons or in the mechanosensory neurons themselves.

Adding to the complexity of this story Wicks and Rankin (1996) compared habituation of the head touch circuit with habituation of the tail-touch circuit by laser ablating the tail and head touch cells respectively. They found that the habituation kinetics of the two sub-circuits of the tap response habituated at different rates. Activation of the head touch neurons led to a more gradual decrement of reversals than in intact animals, while activation of the tail touch neurons led first to sensitization then a small amount of decrement of forward accelerations. The kinetics of habituation of intact worms is an integration of these two curves; subtracting the habituation of accelerations from the habituation of reversals produced a curve very similar to that of intact animals. These data suggest that habituation may be mediated by different mechanisms in the head and tail touch neurons. This is an intriguing notion since work on *Aplysia* (Castellucci and Kandel, 1974) led to the prevailing hypothesis that habituation is mediated by modulation of presynaptic neurotransmitter release. Although this hypothesized mechanism has not been identified, it is what researchers expect to find. The idea of multiple mechanisms underlying habituation within a single organism has not been addressed in many studies.

SHORT-TERM MEMORY FOR TAP HABITUATION

To investigate the cellular basis of tap habituation a candidate gene approach was used. To do this, genes expressed in the sensory neurons of the tap circuit were tested for their role in habituation. Gene expression patterns have been determined for a large number of *C. elegans* genes using beta-galactocidase (Fire et al., 1990) or GFP (Chalfie et al., 1994) transgenes expressed by the promoters of candidate genes.

eat-4

The *C. elegans* homologue of the mammalian glutamate vesicular transporter (VGLUT1), encoded by *eat-4* expressed in the touch cells ALM, AVM, and PLM (Lee et al., 1999) was the first candidate gene to be tested by Rankin and Wicks (2000). Rankin and Wicks hypothesized that if chemical synapses between the touch cells and the interneurons are glutamatergic then mutations in *eat-4* should cause some deficits in habituation to tap. They found that *eat-4* mutants responded normally to the initial tap, however, they habituated significantly more rapidly and to a deeper asymptotic level than wild-type worms (**Figure 2**). In addition, *eat-4* mutants did not show dishabituation after receiving a shock following habituation. Reintroducing the *eat-4* gene in the nervous system of the *eat-4* mutant (rescuing *eat-4*) ameliorated the habituation and dishabituation deficits of the mutant. Their findings supported the hypothesis that neurotransmitter release plays a role in habituation and also may play a role in dishabituation (Rankin and Wicks, 2000).

dop-1

Another gene expressed in the mechanosensory neurons (ALM and PLM) is *dop-1*, which encodes a D1-like dopamine receptor (Sanyal et al., 2004). Because studies in a range of species have indicated that the neurotransmitter dopamine plays a critical role in both vertebrate and invertebrate behavioral plasticity, Sanyal et al. (2004) investigated the role of dopamine as a neural modulator in tap habituation. In these studies the authors analyzed reversal probability and reversal distance separately. They observed that *dop-1* mutants showed a more rapid decline in the number of the worms responding to taps during

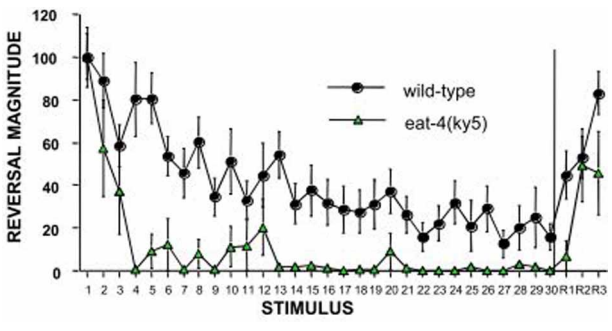

FIGURE 2 | An example of the candidate gene approach: *eat-4* encodes a glutamate vesicle transporter and is expressed on the touch cells. Worms with a mutation in *eat-4* show rapid and complete habituation and slower recovery compared to wild-type worms (Rankin and Wicks, 2000).

habituation training than the wild-type strain. However, reversal distance during habituation for these mutant animals did not show significant differences compared to wild-type worms. Based on these results, they suggested that dopamine might play a role in modulating habituation to tap. This was the first suggestion that reversal rate and reversal distance habituation might be mediated by different mechanisms (Sanyal et al., 2004).

To determine how dopamine modulates tap habituation in *C. elegans*, Kindt et al. (2007) investigated how the DOP-1 receptors modified activity of the mechanosensory neurons. To address this question, they monitored touch-evoked calcium currents in the mechanosensory neurons. While the initial magnitude of the calcium transients in response to mechanical stimulation was the same in the wild-type and *dop-1* mutants, the transient in the ALM neurons of the *dop-1* mutant animals decreased much faster with repeated stimulation than that of wild-type worms. This effect was rescued by expressing *dop-1* in mechanosensory neurons (Kindt et al., 2007). Interestingly, imaging the posterior touch receptor neurons, PLML and R, revealed that the rate of decrement of touch-induced calcium transients was not changed in *dop-1* mutants compared to the wild-type animals. This suggested that dopamine modulated habituation to tap specifically through anterior touch sensory neurons and also that habituation of these cells was mediated, at least in part, by a gradual decrease in cell excitability. To further elucidate the pathway by which dopamine modulated tap habituation they investigated habituation of candidate signal transduction mutants downstream of DOP-1. DOP-1 is a G protein-coupled receptor, so as a downstream candidate they tested Go (*goa-1*) and Gq (*egl-30*) loss-of-function mutants. The *egl-30* mutant showed a very similar phenotype to *dop-1* for habituation. A mutation in *egl-8* (encodes phospholipase C beta, PLC-β, a putative downstream effector of *egl-30*), also had a habituation phenotype similar to *egl-30* and *dop-1* mutants. Hydrolyzation of PIP2 by PLC-β produces DAG and IP3 and in many systems an important effector of DAG is protein kinase C (PKC). PKC-1 is one of three neuronal PKCs in *C. elegans* and a mutation in the gene that encodes this protein caused rapid habituation similar to that seen in *dop-1*, *egl-30*, and *egl-8* mutants. Together these results suggested that *dop-1*, *egl-30*, *egl-8,* and *pkc-1* encode components of a signaling pathway that modulates the mechanosensory response to tap in ALM neurons (Kindt et al., 2007). One of the more interesting aspects of this study was that this dopamine mediated pathway affected habituation only in the presence of food (*E. coli*), the texture of which was detected by the TRP-4 gentle-touch channel in dopaminergic neurons. In the absence of food there were no differences in habituation between these mutant worms and wild-type worms. This suggests that sensory neuron excitability is modulated by the presence or absence of food—an interesting form of sensory plasticity. The dopamine studies also provide support for multiple mechanisms mediating habituation of a response; the behavioral studies showed response probability was altered by mutations in dopamine genes (while reversal distance was not) and the calcium imaging studies showed that ALM responses were modulated by dopamine, while PLM responses were not.

mps-1

Voltage-dependent potassium channels have been shown to contribute in vertebrate and invertebrate learning and memory (Cohen, 1989; Biron et al., 2006). Phosphorylation of these channels through different signaling pathways can modulate excitability of neurons in response to different external events. However, it was recently discovered that this potassium channel may also have auto-enzymatic activity (Weng et al., 2006). The *kcne* genes in humans encode integral membrane proteins which show kinase activity and can modulate voltage gated K^+ channels (KVS-1). MPS-1, a member of the KCNE family, is expressed in ALM and PLM neurons of *C. elegans* (Cai et al., 2009). Cai et al. (2009) investigated whether enzymatic activity of MPS-1 acting on K^+ channels (KHT-1) in mechanosensory neurons play a role in habituation to tap. Applying a single touch to the head or tail of the *mps-1* or *kht-1* mutant animals caused defective backward and forward responses, respectively. The touch response deficit of the *mps-1* mutant could be rescued by expression of wild-type MPS-1 or MPS-1 with an inactive kinase domain. Although they had a wild-type initial response, worms lacking MPS-1 kinase activity habituated more slowly to multiple taps (2, 5, 10, or 60 s ISI; habituation scored as a response magnitude with probability and reversal distance combined), suggesting a critical role of the kinase in the MPS-1 protein for habituation. To explain this result the authors predicted that MPS-1 and KHT-1 form a complex in the touch cells and MPS-1 kinase activity is activated by repetitive stimulation, which results in phosphorylation of KHT-1-MPS-1. Phosphorylation of the KHT-MPS-1 complex decreases K^+ currents, thereby delaying touch neuron repolarization and decreasing the touch neuron excitability by slowing recovery of voltage gated calcium channels required for signal transduction.

Although the first demonstration of habituation in *C. elegans* was published in 1990, and the first gene that played a role in habituation was published in 2000, very few papers have investigated the roles of other genes in habituation. The reason for this slow progress is that most of the experiments tested one worm at a time and then hand scored [or machine scored in Kindt et al. (2007)] one response at a time. Thus, anywhere from 10–40 h were required to run and score habituation for just one strain. Recently a real time computer vision system [Multi-Worm Tracker (MWT)] was designed to allow a high-throughput approach to the study of genes involved in habituation and other behaviors (Swierczek et al., 2011). The MWT is able to analyze image data in real time and monitor many worms on a single plate using a high-resolution camera. Swierczek et al. (2011) used the MWT to conduct a pilot screen for novel genes involved in tap habituation. 33 strains with mutations in genes involved in a variety of predicted functions were habituated and the probability of reversal and reversal distance were analyzed. Among the strains tested was a mutant isolated in a screen for chemosensory adaptation, *adp-1* (Colbert and Bargmann, 1995). Similar to their behavior in the presence of persistent gustatory and olfactory stimuli, *adp-1* mutants maintained robust responding following repeated taps, suggesting a common mechanism between modalities. Chemosensory defective cilia mutants (e.g., *che-2*) also displayed altered tap habituation. The rapid habituation of these mutants may reflect an inability of the dopamine neurons

to sense the texture of the bacterial food source and signal to *dop-1* on ALM, but reversal magnitude phenotypes not seen in the absence of food suggests that chemosensory neurons may modulate the tap response. Finally, loss of tomosyn ortholog, *tom-1*, resulted in very rapid and deep habituation (**Figure 3**). TOM-1 is involved in neurotransmitter release, perhaps linking it to the *eat-4* mutant phenotype. These data demonstrate the usefulness of the MWT as a tool to elucidate mechanisms of tap habituation (Swierczek et al., 2011).

Taken together, these studies suggest that short-term habituation is not as simple as has been thought. These data indicate that habituation is not a unitary process; response magnitude and response probability are mediated or modulated by different genetic pathways. In addition, the head sensory neurons show different patterns of decrement from the tail sensory neurons and are differentially modulated by dopamine. This suggests that different cellular processes may be involved in habituation in different neurons. We also see that short-term habituation is modulated by the presence of food. These studies show that there are increasingly complex aspects to the "simplest form of learning."

LONG-TERM MEMORY FOR TAP HABITUATION

Long-term memory (LTM) is defined as the process of saving information in the nervous system that is retrievable over a long period of time. Studies in a number of organisms ranging from human (Ebbinghaus, 1885) to *Aplysia* (Carew et al., 1972) to *Drosophila* (Tully and Quinn, 1985) have indicated that spaced training, in which there are rest periods during training, produces better LTM than massed training (same number of stimuli with no breaks). LTM for habituation of the tap withdrawal response was first shown by Rankin et al. in 1990, and the protocol was replicated and modified in Beck and Rankin (1995) and Rose et al. (2002). The optimal protocol consisted of four blocks of training (1 h rest between each blocks) followed by one block of testing 24 h later. Control groups received one tap at the end of training and one block of test stimuli after 24 h (Rose et al., 2002). Rose et al. showed that as the number of training blocks increased from 1 to 4, the amount of memory also increased. Interestingly, using a 40 min inter-block interval during the training instead of 1 h diminished the significant difference between the control and trained group. Beck and Rankin (1995) showed that applying 45 min of heat shock in the inter-block intervals to block ongoing protein synthesis eliminated memory 24 h later. Heat shock applied in the first or second 15 min in the rest intervals blocked memory, however, if applied for 15 min, 30 min into the block it did not affect memory. Together these data suggest that the first 30–40 min after each block are critical times for memory formation.

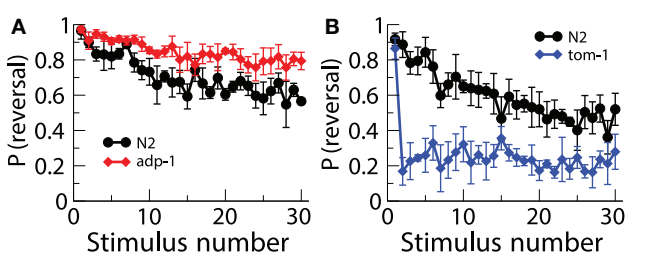

FIGURE 3 | Using the multi worm tracker to test a number of strains of worms uncovered new mutations affecting habituation. There was lower response probability habituation to tap in *adp-1* worms **(A)** and greater response probability in *tom-1* worms **(B)** (Swierczek et al., 2011).

A candidate gene approach was used to investigate mechanisms of LTM for tap habituation. Rose et al. (2002) investigated the function of EAT-4, a vesicular glutamate transporter shown to play a role in short-term tap habituation (Rankin and Wicks, 2000) in LTM of tap habituation. Mutations in *eat-4* result in a decrease in glutamate available at the sensory neuron terminal. In *eat-4* worms, spaced training with a tap stimulus did not produce LTM, however, Rose et al. (2002) observed that distributed training with a stronger stimulus, a train of taps produced LTM in *eat-4* mutants. They suggested that stronger stimuli are necessary to release sufficient glutamate from the mechanosensory neurons to produce LTM for habituation in *eat-4* mutants. Based on these data they hypothesized that presynaptic glutamate release from the sensory neurons is necessary for LTM formation (Rose et al., 2002). The necessity of presynaptic glutamate release suggests that postsynaptic glutamate receptors might play an important role in LTM for habituation. GLR-1 is a receptor subunit of a non-NMDA excitatory ionotropic glutamate receptor subtype expressed in the tap circuit interneurons (AVA, AVB, AVDs, and PVC; Hart et al., 1995; Maricq et al., 1995). Although *glr-1* mutants showed normal short-term memory at a 60 s ISI, when tested for LTM of habituation, *glr-1* mutant trained worms were not significantly different from the control group suggesting that *glr-1* was required for LTM formation. To support this hypothesis, a pharmacological experiment in which worms were exposed to DNQX, a non-NMDA-type glutamate receptor antagonist, showed that worms trained in the presence of DNQX did not exhibit LTM. Thus, activation of GLR-1 receptors is critical for memory (Rose et al., 2002).

In *Aplysia* long-term habituation is mediated by a down regulation of vesicles in the siphon sensory neurons (Bailey and Chen, 1991). In mammals LTD (a lasting decrease in synaptic strength similar to long-term habituation) is mediated by down-regulation of AMPA-type glutamate receptors on the post-synaptic neurons. To determine whether LTM training for habituation was associated with changes in the sensory neurons or the command interneurons, worms carrying transgenes with green fluorescent protein fused to pre- and post-synaptic markers were given long-term habituation training and then imaged. One strain expressed GFP marking a synaptic vesicle protein, synaptobrevin (*snb-1*), in the mechanosensory neurons (Nonet, 1999). The other expressed GFP tagged GLR-1 receptor subunits (Rongo and Kaplan, 1999). The results showed no presynaptic changes in GFP tagged *snb-1*, however, spaced training for LTM induced a reduction in the size, but not the number, of GLR-1::GFP puncta in the posterior ventral nerve cord (**Figure 4**) Rose et al. (2003). Based on these results Rose et al hypothesized that the size of synapses was reduced by memory training, however, the number of synapses was not changed. Interestingly, with more training blocks over several days, decreases were seen in synaptbrevin GFP in the sensory neurons suggesting that plasticity in the sensory neurons

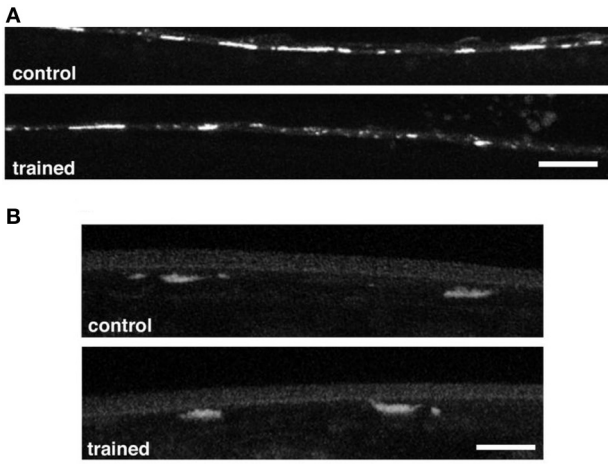

FIGURE 4 | (A) Representative images of GLR-1::GFP expression in interneurons of the posterior ventral nerve cord in a worm that had been given spaced training for long-term memory for habituation 24 h before and an untrained control worm. There were significantly smaller GFP clusters in the trained worms than in the control worms. **(B)** Representative images of the vesicles in tap sensory neuron terminals visualized with a synaptobrevin GFP marker in control and trained worms. There was no difference in measured GFP expression between the trained and control worms (Rose et al., 2003).

requires more stimulation than plasticity of glutamate receptor trafficking in the command interneurons.

INTERMEDIATE MEMORY FOR TAP HABITUATION

In many biological models of learning and memory, researchers have found that, in addition to short-and long-term forms of memory, they also see various forms of memory that they call intermediate memory. These forms of memory do not seem to require protein synthesis and do not last as long as LTM. In *Drosophila* a protein synthesis-independent form of memory (Anesthesia Resistant Memory; ARM) is seen after massed training (Tully and Quinn, 1985). To determine whether massed training could result in an intermediate form of memory in *C. elegans*, Li et al. (2013) looked for the presence of intermediate memory by testing worms 12–16 h after massed training with 80 tap stimuli at a 60 s ISI (No LTM was observed 24 h after massed training, Rose et al., 2002). They found that 12–16 h after massed training, worms did show a significant decrease in reversal magnitude compared to control worms. Thus, intermediate term memory lasts 16 but not 24 h. Investigations into the mechanisms governing this intermediate memory revealed that, unlike LTM, intermediate memory did not require the *glr-1* glutamate receptor subunit. Intermediate-term memory was not affected by applications of heat-shock to block protein synthesis following training (Li et al., 2013).

In order to determine what effect intermediate-memory training had on the tap-withdrawal circuit, worms carrying the GFP tagged *snb-1* expressed in the mechanosensory neurons were given 12 h memory training. Changes in the intensity of the GFP signal would indicate alterations in expression of *snb-1* at the presynaptic terminal. Li et al. (2013) found that, 12 h after habituation training, expression of SNB-1::GFP was significantly higher in trained animals compared to controls. This alteration in the presynaptic terminal was not present 24 h later suggesting that, like the behavioral expression of memory, the modulation at the synapse is transient. These data suggested that 12 h after massed training there was an increase in synaptic vesicles in the sensory neuron terminals. The question then was what is in those vesicles? Normal 12 h memory was found in *glr-1* and *eat-4* mutants indicating that glutamate was not critical for intermediate memory. Using a candidate gene approach, Li et al. (2013) then investigated whether an inhibitory neuropeptide was released by the touch cells. *flp-20* encodes a FMRFamide-related short peptide neurotransmitter precursor with unknown function. *flp-20*, along with *flp-4* and *flp-8*, are the only known peptide precursor genes expressed in the mechanosensory cells (Kim and Li, 2004). Intermediate-memory massed training was given to *flp-4, flp-8,* and *flp-20* mutants and the results showed that only *flp-20* was required for the formation of intermediate memory. Indeed, *flp-20* worms did not show intermediate memory 12–16 h after training, and in a *flp-20* background there was no increase in SNB-1::GFP expression in the presynaptic terminals. Reintroduction of *flp-20* in the mechanosensory neurons rescued the behavioral expression of intermediate memory for massed training. This effect is specific to intermediate memory as *flp-20* mutants were capable of forming LTM after spaced training. Thus, as in *Drosophila*, in *C. elegans* different stimulus paradigms recruit different and independent mechanisms of lasting memory.

SHORT- AND LONG-TERM CONTEXT CONDITIONING IN TAP HABITUATION

An organism is not usually exposed to a single stimulus, rather stimuli from multiple sensory modalities occur within a complex environment. Is an animal with a nervous system as small as *C. elegans* able to integrate information across sensory modalities? Historically, simple forms of learning have been divided to two types: associative and non-associative. Habituation, based on this traditional system of classification was placed in the non-associative learning category (Rankin, 2000). However, several studies have suggested that environmental cues have an effect during *C. elegans* training and retrieval of the memory for habituation (Rankin, 2000; Lau et al., 2013). Rankin (2000) showed that worms trained and tested in the presence of a unique context cue (the taste of sodium acetate) showed greater retention of tap habituation 1 h after training compared to a group that did not receive a consistent cue at training and testing. This suggested that worms were capable of associating a chemosensory cue with tap habituation such that after training in the salt taste, the presence of the salt taste cued their habituation memory and test responses were smaller. This is context conditioning for habituation. Lau et al. (2013) replicated the taste context conditioning and extended it to smell, showing that worms trained and tested in the presence of a unique odor cue showed enhanced memory compared to worms that did not receive the cue at both training and testing. Lau et al. used a candidate gene approach to determine whether they could genetically distinguish short-term and LTM for habituation and for context habituation in

C. elegans. They chose three candidate genes: *crh-1* (homolog of transcription factor, Cyclic-AMP response element binding protein; CREB), *glr*-1 (non-NMDA-type glutamate receptor subunit), and *nmr-1* (NMDA-type glutamate receptor subunit).

Short-term context conditioning experiments were performed by giving 30 taps at a 10 s ISI (habituation training) in the presence or absence of the odorant diacetyl (cue and no cue groups). Because the odorant was on the lid of the Petri plate it could be removed immediately after training. Following a 1-h break, worms were again exposed to the appropriate context (cue or no cue) and given the test stimuli. For LTM experiments, worms were exposed to their randomly assigned context for spaced training (4 or 6 blocks of 20 stimuli at 10 or 60 s ISI with 1 h rest between blocks). Between blocks the plate lids were changed so that worms were not exposed to the odorant during the inter-block interval. 24 h after training worms were tested with 5 taps at a 10 or 60 s ISI either in the presence of the odorant or not. In *C. elegans*, LTM for tap habituation is produced by applying 4–5 blocks of 20 stimuli at 60 s ISI and memory is not detectable if a 10 s ISI is used. Normal LTM for habituation at a 60 s ISI was not enhanced by a context cue, possibly because the 20 min long exposure on each block was enough to produce sensory adaptation, decreasing the salience of the odor cue. Interestingly, LTM for habituation at a 10 s ISI was apparent if worms were trained and tested in the same context. This suggested that the combination of the two sensory modalities produced a new kind of memory not present with the single training cue.

The genetic analyses of short and long-term habituation and context dependent habituation showed that each of the genes tested had a different pattern of results (**Figure 5**). CREB has been shown to be critical for LTM in various species (e.g., Bernabeu et al., 1997; Josselyn et al., 2004) and worms with a mutation in *crh-1* (homolog of CREB in *C. elegans*) show normal short-term habituation but no LTM (Timbers and Rankin, 2011). For context conditioning experiments, Lau et al. found that worms with a mutation in *crh-1* showed short-term habituation and short-term context conditioning but no long-term context conditioning. Rescuing CRH-1 in mutant worms rescued the long-term context conditioning deficit. In previous studies, it was shown that *glr-1* (encoding a non-NMDA-type glutamate receptor subunit) is critical for LTM performance, but not necessary for short-term memory (Rose et al., 2002). Lau et al. found that in addition to not showing long-term habituation, *glr-1* mutants did not show short-term or long-term context conditioning. *nmr-1* encodes an NMDA-type glutamate receptor subunit broadly expressed in interneurons of the mechanosensory circuit in *C. elegans*. Lau et al. (2013) showed that an *nmr-1* mutant did not show any deficit in short-term or LTM for habituation, however, NMR-1 was critical for both short- and long-term context effects in habituation. How is the input from the two sensory systems integrated? Previous studies showed that expression of *nmr-1* in the pair of RIM interneurons was essential for starvation and taste association learning (Kano et al., 2008). Based on this, Lau et al. (2013) investigated the role of *nmr-1* in RIM interneurons by cell-specific rescue of this gene and found that expression of NMR-1 in the RIM interneurons was sufficient to rescue both short- and long-term context conditioning in *nmr-1* mutant animals.

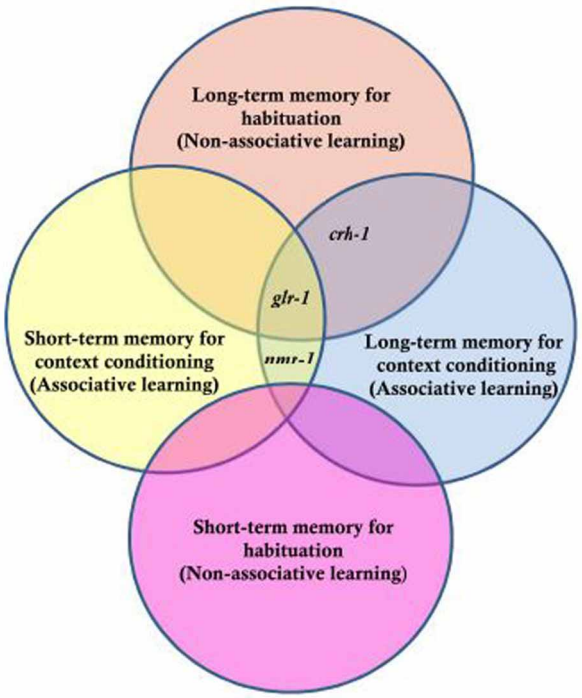

FIGURE 5 | Genes tested for their role in long- and short-term memory for non-associative habituation and associative context conditioning. Three genes were tested, *crh-1* which is the worm homologue of CREB, *glr-1* the worm homologue of a glutamate AMPA-type receptor subunit and *nmr-1* the worm homologue of an NMDA-type glutamate receptor subunit. The results indicated that *nmr-1* is required for short and long-term context conditioning but not necessary for non-associative learning; *crh-1* is critical for long-term but not short-term memory for both associative and non-associative memory and that *glr-1* is not required for short-term non-associative learning, but required for long term as well as short- and long-term context conditioning [Based on data from Lau et al. (2013)].

This suggested that the interneuron RIM is a key site of integration for sensory input from chemosensory and mechanosensory neurons. Context conditioning learning in the mechanosensory circuit of *C. elegans* offers a new opportunity to investigate the genes critical for short- and long-term associative and non-associative learning as well as to study how sensory integration is encoded in the memories.

These experiments offer another example about how complex habituation can be, even in an animal with only 302 neurons. *C. elegans* behavior can be affected by previous experience and the response to tap can be altered by the memory of previous training. *C. elegans* is also capable of processing information from multiple sensory systems simultaneously and of forming an association of those stimuli that influences later memory. For short-term habituation, a chemosensory context cue enhances the memory of habituation training 1 h before. For LTM for context conditioning, worms show memory after a tap protocol that would not normally lead to memory. This suggests that the association of tap habituation in the presence of a chemosensory cue led to production of a novel type of memory. In mammals, multiple cues often lead to better memory formation than a single

cue (Walker et al., 2005; Marschner et al., 2008). These findings suggest that in every non-associative learning paradigm there is the potential for associative learning to be occurring, as well as for the effects of previous training to influence the behavior. Once again the simplest form of learning gets additional layers of complexity.

DEVELOPMENTAL EXPERIENCE DEPENDENT SENSORY PLASTICITY

So far, this review has focused on the behavioral characteristic and molecular mechanism of short-term, intermediate-term and LTM in adult *C. elegans*. However, mechanosensory stimulation during development can also have a profound effect on adult sensory systems and behavior (Rose et al., 2005). As in mammals (Diamond et al., 1966), activity-dependent processes shape the final patterns and strengths of synaptic connections in the worm's nervous systems (Peckol et al., 1999; Zhao and Nonet, 2000; Rose et al., 2005).

In *C. elegans,* neural wiring and connectivity has been described and was originally suggested to be invariant between individuals (White et al., 1986), nevertheless further research has revealed that activity in the nervous system might be necessary for normal axonal branching and neuronal morphology (Peckol et al., 1999; Zhao and Nonet, 2000). Rose et al. (2005) examined the influence of mechanosensory stimulation that comes from contacts with other worms during development. In the laboratory worms are usually grown in groups in small Petri plates, however, in this experiment, worms were raised either in groups (colony condition) or one worm to a plate (isolated condition). When 4 day-old worms were examined, those that had been raised in the isolated condition showed significantly smaller responses to a single tap compared to the worms that developed in a group. To test the possibility that isolation affected sensory systems in general, Rose et al. looked at different behaviors mediated by the same interneurons and motor neurons as the tap withdrawal circuit. The magnitude of the reversal response to a heat probe was indistinguishable between isolate and colony-reared worms. This suggested that the decrease in reversal behavior to tap in isolated worms resulted from the lack of mechanical stimulation during larval development. Consistent with this hypothesis, thirty tap stimuli administered at any larval stage reversed the effect of isolation on the tap response in adult worms (Rose et al., 2005; Rai and Rankin, 2007).

Rose et al. (2003) found that the GLR-1 glutamate receptor subunit was critical for LTM for habituation training in adult worms. Based on this finding, Rose et al. (2005) hypothesized that GLR-1 function might be important for the effects of isolation on the tap-withdrawal response. To test whether *glr-1* played a role in the decreased response to tap in isolated worms, the magnitudes of reversal responses to tap for the colony and isolate groups were measured in *glr-1* mutants and in a transgenic strain in which *glr-1* expression had been rescued with the endogenous promoter. There were significant differences in response magnitudes between isolate- and colony-raised worms in the wild-type strain and the *glr-1* rescue strain, however, isolated- and colony-raised *glr-1* mutants did not show any difference in the reversal response to tap. Based on these results, it appeared as though the decreased response to tap seen in isolated worms was mediated by the *glr-1* type glutamate receptor subunit.

Rose et al. (2005) used GFP to show that changes in the expression of *glr-1* accompanied the formation of LTM for habituation in adult *C. elegans*, while changes were not seen in the expression of *snb-1* in the mechanosensory neurons. The same GFP constructs were used by Rose et al. (2005) to determine whether there were anatomical changes underlying the effect of isolation on the response to tap. To test whether the amount of stimulation during development altered the expression of *glr-1* receptors, Rose et al. again imaged the GLR::GFP strain (Rongo and Kaplan, 1999). Similar to what was seen in adult memory they found that the number of the GLR-1::GFP clusters in isolate-reared worms was not different from colony-reared worms, however, the size of the clusters was significantly reduced. As with the behavior, brief stimulation (30 taps) during any larval stage was sufficient to increase the level of *glr-1* expression in the interneurons of isolated worms (Rose et al., 2005; Rai and Rankin, 2007). Rose et al. also examined transgenic colony and isolate worms expressing *snb-1*-GFP (SNB-1::GFP; Nonet, 1999), in the touch cells. The expression of SNB-1 in isolated-raised worms was lower than expression in colony-raised worms, which suggested that the number of synaptic vesicles in the mechanosensory neuron terminals was reduced. Thus, mechanosensory deprivation during development appeared to lead to the production of weaker synapses with fewer vesicles in the sensory neurons and fewer receptors on the interneurons than in colony reared animals. Synabtobrevin expression in isolated worms could only be rescued by brief mechanical stimulation applied at the first larval stage (L1) (Rai and Rankin, 2007). Rai and Rankin (2007) showed that as worms aged, progressively more mechanosensory stimuli were required to rescue *snb-1* expression in isolate reared worms to colony-reared levels (i.e., 400 stimuli in L2 or L3 and 800 stimuli in L4 and adult). Again, these results suggest that trafficking of glutamate receptors responds more rapidly to experience than does a presynaptic vesicle protein. The results of these experiments also revealed a strong association between rescuing the response to tap and postsynaptic *glr-1* expression in isolated animals. This suggested that tap reversal behavior is tightly related to glutamate receptor expression. In other words, changes in glutamate receptor expression may underlie the observed changes to the behavioral response to tap (Rai and Rankin, 2007).

The study on the role of early experience on the tap response also led to insight into the mechanisms governing the rate of habituation. When isolate-reared worms were given 30 mechanosensory stimuli at any stage of development the initial response to tap and the level of *glr-1* were similar to colony-reared worms, however, this "rescue" was deceptive, as the "rescued" worms habituated differently than the colony-reared worms. Three groups of adult worms were given habituation training: an isolated group, a colony group, and a rescued isolated group that had been given sufficient stimulation during larval development to rescue the GLR-1::GFP, but not the *snb-1* GFP (Rose et al., 2005). The results showed that the isolate group had significantly smaller initial responses to tap than either the colony

or the rescued isolate group, while the rescued isolate and the colony groups were not different from one another. However, with repeated taps, the rescued isolate group showed much more rapid decrement than did the colony group. In fact, the rescued isolates habituation curves looked very like the habituation curves for the *eat-4* worms (**Figure 2**). The isolated worms had lower *snb-1* expression in the terminals of their touch cells and similarly, *eat-4* worms had a defect in the loading of glutamate in vesicles, suggesting they also had less glutamate available for release. These data suggest the hypothesis that regulation of neurotransmitter release plays an important role in regulating rate of response decrement during habituation. This hypothesis is supported by the finding that another gene involved in regulating neurotransmitter release, *tom-1*, also showed a similarly altered rate of habituation to tap (Swierczek et al., 2011).

CHANGES IN TAP HABITUATION WITH AGING

In addition to experience dependent changes during development, the ability to learn and remember also alters with normal aging. Beck and Rankin (1993) studied aging *C. elegans* (10 and 12 days post-hatching) and found that they habituated more rapidly and showed less spontaneous recovery than middle-aged adults. This is not unexpected as *C. elegans* rapidly begins to show nervous system and muscle breakdown after it has passed its reproductive prime (Pan et al., 2011). However, what about during middle age, before the neurons and muscles show evidence of degeneration? Are there changes in sensory plasticity during this period? Worms develop from fertilized egg to reproductive young adult in 3.5 days; they then are reproductive adults for 3–4 days, before becoming senescent (Wood et al., 1980). Timbers et al. (2013) investigated whether there were age-dependent changes in habituation in middle-aged *C. elegans*. They tested the effect of age on tap habituation in populations of worms 72 (day 0 of adulthood), 84, 96, 108, and 120 (day 2.5 of adulthood) h old. They found that habituation of reversal probability to tap in young animals (72 h old) occurred more slowly than for older adult (120 h) animals (**Figure 6**). The factors responsible for this difference between 72 and 120 h old worms might be in the transduction of the tap stimulus or might reflect some change in cell excitability or synaptic strength as worms aged. To investigate this Timbers et al. used transgenic worms expressing Channelrhodopsin-2 (blue light-gated cation channel) in the touch cells (Nagel et al., 2003; Boyden et al., 2005). Applying a short pulse of blue light to the transgenic worms activates the touch cells and induces a reversal response similar to that seen following a mechanical tap. If activation of the touch cells by blue light showed the same results as applying taps in adult worms (72, 84, 96, and 120 h old), the age-dependent changes in behavior must originate downstream of sensory transduction. However, the results of this experiment showed that age was not related to the probability of responding to repeated blue light pulses, suggesting that the age-dependent changes in behavior originate upstream of cellular depolarization. A known characteristic of habituation is that less intense stimuli cause more habituation, whereas more intense stimuli cause less habituation (Thompson and Spencer, 1966; Groves and Thompson, 1970; Rankin et al., 2009). Consistent with this body of literature, Timbers et al. (2013) also showed an association between stimulus intensity and habituation of young adult worms (72 h-old), however, older worms showed no such association. Thus, the rate of reversal probability habituation is dependent on the ability to discriminate stimulus intensity, which the younger worms seem to do to a greater extent than the older worms. Possible explanations for the decrement in stimulus discrimination with age include changes in

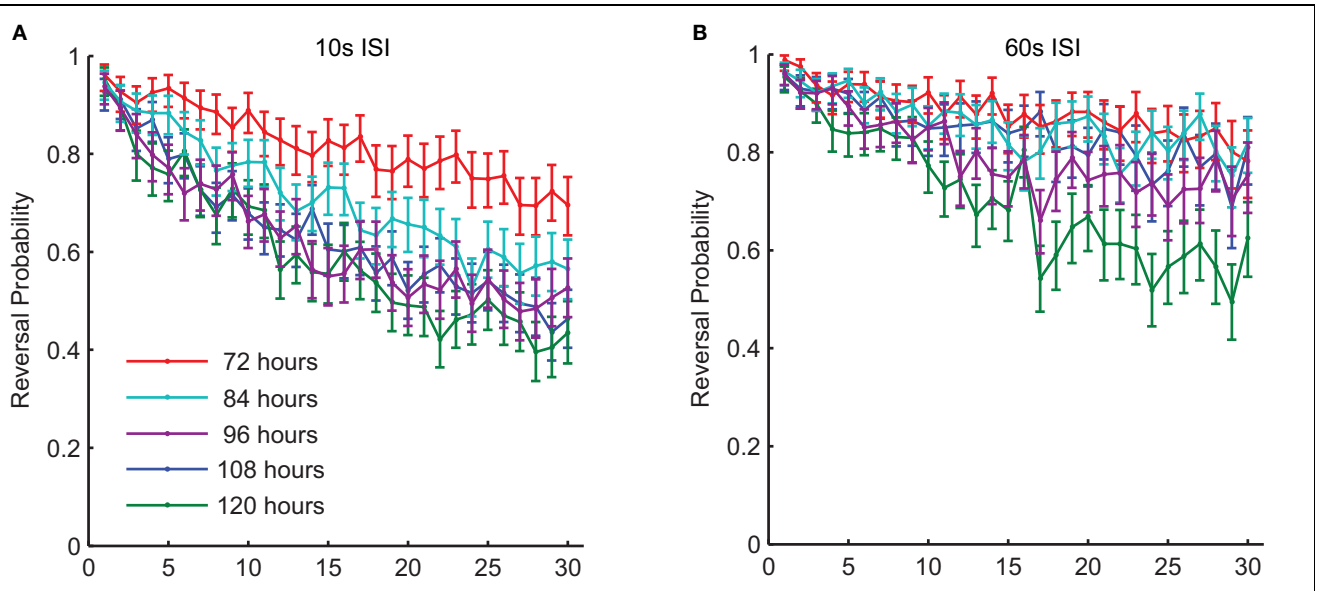

FIGURE 6 | There were changes in habituation of response probability throughout middle-age in wild-type worms. This graph shows response probability habituation for 72-, 84-, 96-, 108-, and 120-h-old (all reproductive adults) wild-type *C. elegans* in response to a series of 30 taps. Age was significantly related to the probability of responding to the final tap at both a 10-s ISI **(A)** and at a 60-s ISI **(B)** (Timbers et al., 2013).

neuronal excitability, changes in the thickness of the cuticle or in the strength of the connection of the mechanosensory cells to the body wall (Timbers et al., 2013).

CONCLUSIONS

Despite its apparent simplicity, the *C. elegans* tap withdrawal response is a complicated behavior that shows a great deal of plasticity. External mechanical stimuli activate forward and backward sub-circuits and imbalanced activity of the forward and backward sub-circuits determine the direction of the movement. This response is experience dependent and plasticity of the circuit can alter behavior for days. Thus, far tap habituation has shown short- intermediate and LTM—a surprising catalog of types of memory for an organism that only lives a few weeks. A number of factors can influence habituation and the memory for habituation, these include the presence or absence of food, the presence of absence of contextual cues, the amount of early mechanosensory stimulation that particular worm had during larval development, and the age of the worm at the time of testing. In addition different neurons in the circuit are affected by different factors and habituate at different rates. The search for the mechanisms underlying habituation will have to take all of these variables into account, rendering this so-called "simple" form of learning not so simple after all.

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Developmental and activity-dependent plasticity of filiform hair receptors in the locust

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A group of wind sensitive filiform hair receptors on the locust thorax and head makes contact onto a pair of identified interneuron, A4I1. The hair receptors' central nervous projections exhibit pronounced structural dynamics during nymphal development, for example, by gradually eliminating their ipsilateral dendritic field while maintaining the contralateral one. These changes are dependent not only on hormones controlling development but on neuronal activity as well. The hair-to-interneuron system has remarkably high gain (close to 1) and makes contact to flight steering muscles. During stationary flight in front of a wind tunnel, interneuron A4I1 is active in the wing beat rhythm, and in addition it responds strongly to stimulation of sensory hairs in its receptive field. A role of the hair-to-interneuron in flight steering is thus suggested. This system appears suitable for further study of developmental and activity-dependent plasticity in a sensorimotor context with known connectivity patterns.

Keywords: insect flight, filiform hair receptors, wind receptors, developmental plasticity, interneuron

INTRODUCTION

To serve the requirements of behavior in different life stages and different biological habitats, the nervous system must exhibit a considerable degree of flexibility, particularly in holometabolous insects. In the tobacco hawkmoth, *Mandua sexta*, for example, larva and adult exhibit different life history traits associated with their respective functions, occupy different ecological niches and show different behaviors. The predominant larval behaviors are crawling, feeding, defensive behaviors, and moulting, whereas in adults these are walking and flying, feeding, and all behaviors associated with courtship and reproduction. These changes in the nervous system are induced by and dependent on developmental hormones including ecdyson and juvenile hormone, and they occur predominantly at pupation and during metamorphosis (Riddiford et al., 2003). For motor neurons that persist from larva to adult and innervate muscles that have very different contractile properties in these two life stages, it has been well established that dendritic morphologies and electrical properties change markedly during development (Kent and Levine, 1988; Levine and Weeks, 1990; Duch and Levine, 2000; Tissot and Stocker, 2000; Weeks, 2003). Not only hormones but also neuronal activity has a role in this developmental plasticity (Duch and Mentel, 2003).

Such changes are not restricted to the motor system. Some persisting sensory receptors also exhibit structural changes with respect to their axonal arbors (Levine et al., 1985; Kent and Levine, 1988; Kent et al., 1996; Tissot and Stocker, 2000). In general, these changes follow similar patterns as observed in motor neuron dendrites: retraction of larval axonal branches is followed by a more elaborate outgrowth to generate the adult axonal arbors. However, the changes in the sensory axonal arbors are less conspicuous than those in the motor neuron dendrites. Corresponding changes are observed in the relevant sensory-motor circuitry (Gray and Weeks, 2003).

In hemimetabolous insects such as locusts, corresponding developmental changes are less obvious. The first nymph already seems like a miniature version of the final adult animal, except for the missing wings that develop postembryonically. In these insects most structural changes during development would thus appear to be associated with wing development and with the subsequent commencement of flight behavior (e.g., Altmann et al., 1978). Nonetheless, as the insect grows and expands its body surface, sensory cells are added virtually everywhere, particularly mechanosensory hairs. This has been studied probably in most detail in the cerci of crickets (Murphey, 1986; Dangles et al., 2006, 2008; Mulder-Rosi et al., 2010; Miller et al., 2011), with regard to their endowment with wind sensitive hairs. Gradual changes in the strength and localization of synaptic contacts are essential here to accommodate the increasing number of sensory cells impinging on a given central nervous interneuron. These changes appear relatively small, however, compared to the complete retraction and new outgrowth of whole neuronal arbors during metamorphosis.

Here, we present a model system in the locust that allows study of developmental plasticity in sensory projections and connectivity. Wind sensitive hairs on the head and especially the thorax make monosynaptic connections to an identified interneuron,

A4I1. These sensory hair-to-interneuron connections changes during nymphal development, and these changes depend on neuronal activity with regard to both morphology and synaptic contact. We further present a first experiment addressing possible functions of this sensory hair-to-interneuron system in locust flight control.

RESULTS AND DISCUSSION

THE FILIFORM HAIR SYSTEM OF THE LOCUST PROTHORAX AND HEAD

Filiform hairs are extremely sensitive to wind—air current—or to local movement of air particles—low-pitched sound and infrasound—such as occur in the near field of a loudspeaker. Filiform hairs are known from the cerci of many insects, such as cockroaches and crickets (Murphey, 1986; Dangles et al., 2006, 2008; Mulder-Rosi et al., 2010; Miller et al., 2011), and from caterpillars, where they mediate escape responses (Tautz and Markl, 1978; Gnatzy and Tautz, 1980; Blagburn and Beadle, 1982; Pflüger and Tautz, 1982; Bacon and Murphey, 1984; Ogawa et al., 2006; Heys et al., 2012). Spiders possess similar, highly sensitive hair sensilla known as trichobothria. They are also involved in escape responses (Gronenberg, 1989) and in prey capture as well (Barth et al., 1995).

These filiform hairs enable insects and spiders to detect wind from the wing beat of predatory wasps or even the wind puff produced by the protruding tongue of a striking toad (cited in Camhi, 1984). The individual filiform hair exhibits clear directional sensitivity (Dagan and Volman, 1982). Due to the spatial arrangement of hairs with different directional preferences on the cercus of a cricket or cockroach, stimuli from all directions are detected by the ordered array of receptors, and stimulus direction is coded accordingly (Murphey, 1986; Dangles et al., 2006, 2008; Mulder-Rosi et al., 2010; Miller et al., 2011). The synaptic connections between these hairs and first-order interneurons are remodeled during postembryonic development (Chiba et al., 1988), although in a more gradual fashion than during metamorphosis in holometabolous insects. Such remodeling in hemimetabolous insects may nonetheless be profound.

Less well known than cercal hairs are similar wind sensitive filiform sensilla on other body parts. In locusts, these occur on the frontal head and on the thorax, namely, on the ventral probasisternum, the lateral proepisternum, and the dorsal pronotum. In the first nymphal instar, there are 8 hairs on each half of the probasisternum, 2 on each proepisternum, and 3 or 4 on each half of the pronotum (**Figure 1A**, red arrows point to the hair receptors). During each moult new hair receptors are added, resulting in a total number of about 300 probasisternal cuticular hairs in the adult (Pflüger et al., 1994). **Figure 1C** shows a scanning electron micrograph of the adult probasisternum with its arrangement of filiform hairs. A single mechanosensory cell with its dendrite attached to the base of the hair shaft is revealed by a silver intensified cobalt chloride fill (Watson and Pflüger, 1984) in **Figure 1B**.

The filiform hairs that are present in the first nymphal instar are easily recognized in adults by their relative positions, and most conspicuously by the lengths of their hair shafts which are the longest compared to all other hairs. In addition, these are the receptor cells most sensitive to wind stimuli in adults (Pflüger and Tautz, 1982). Thus, individual filiform hairs can be monitored throughout postembryonic development.

The above mentioned hairs (**Figure 1A**) are part of the receptive fields of a (bilaterally symmetric) pair of projection neurons (A4I1, **Figure 1D**, schematic drawing; Pflüger, 1984), and all make monosynaptic connections within the prothoracic ganglion (Burrows and Pflüger, 1990; see also **Figure 2**). Some of the output connections of this projection neuron (A4I1) are described below.

THE CENTRAL PROJECTIONS OF FILIFORM HAIRS EXHIBIT STRUCTURAL DYNAMICS IN POSTEMBRYONIC DEVELOPMENT

The central axonal arbor of an individual filiform hair was stained by placing a blunt glass microelectrode filled with a solution of either cobalt salts or fluorescent dyes over the base of the cut hair shaft and applying currents for up to 45 min. In adult locusts (**Figure 2B**; see Pflüger and Burrows, 1990), the projection patterns of probasisternal hairs exhibit exclusively contralateral projections (**Figure 2**, red) whereas both the proepisternal and pronotal receptors have only ipsilateral projections (**Figure 2**, blue). In the first nymphal instar (**Figure 2A**; Pflüger et al., 1994), by contrast, the axonal arbors of the same probasisternal filiform hairs show both ipsi- and contra-lateral projections (**Figure 2A**, red), whereas those of proepisternal and pronotal hairs only reveal ipsilateral projections, like in the adult. When individual probasisternal hairs were stained in the different nymphal instars, those that were at the most lateral position of the probasisternum lost their ipsilateral axonal branch first whereas those at the most median position lost their ipsilateral branch latest, i.e., only in the final nymphal instar before the imaginal moult. Thus, there is a temporal gradient of loss of the ipsilateral branch in the projection pattern that parallels the position of the hair on the probasisternum from lateral to median. There is an increasing loss of second and higher order branches in the ipsilateral axonal arborization, and at the same time complexity of branching on the contralateral side increases in the course of consecutive nymphal instars. In contrast, proepisternal and pronotal hairs exhibit ipsilateral projections throughout all nymphal instars, and appear to undergo only synaptic refinement and pruning within the general layout of this ipsilateral branch (Pflüger et al., 1994).

In order to study the contribution of activity-dependent processes to this developmental plasticity, the activity of a proepisternal filiform hair receptor was blocked in all postembryonic stages—nymphal instars—by either immobilizing the hair shaft by wax or by cutting it close to its base immediately after each nymphal moult (**Figure 2C**, red X). These experimental procedures interfered only with the neuronal activity generated by the mechanoreceptor associated with the respective hair but not with the position of the hair on the cuticle. Subsequently, in adult locusts, the projection patterns of the manipulated hair were examined, as well as those of the adjacent untreated probasisternal filiform hairs, and those of the contralateral probasisternum were used as controls. Compared to normal development, the manipulated hair exhibited sparser arborizations. Most notably however was the fact that the filiform probasisternal hairs adjacent to the manipulated proepisternal hair retained their ipsilateral branches (**Figure 2C**). The controls on the untreated body side

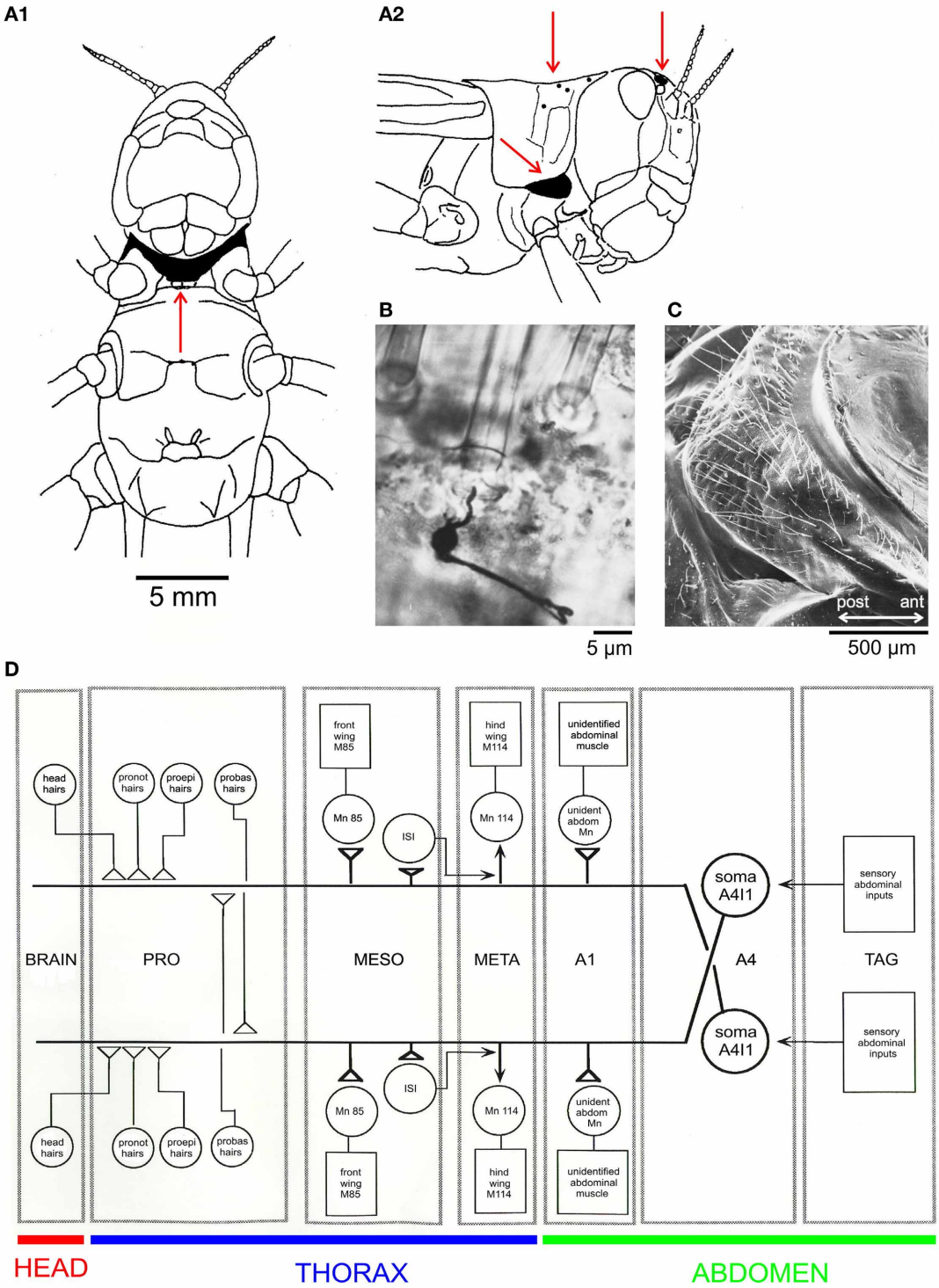

FIGURE 1 | The locust filiform hair-to-interneuron system. (A) Schematic drawings of a locust viewed from the ventral **(A1)** and lateral **(A2)** sides; red arrows indicate locations of filiform hairs in the areas shaded in black: the ventral probasisternum **(A1)**, the lateral proepisternum (**A2**, ventral), the dorsal pronotum (**A2**, dorsal), and field 1 of the wind sensitive head hairs. **(B)** Silver-intensified cobalt fill of the peripheral sensory nerve revealing cell body and initial axon segment of a mechanoreceptive sensory neuron and its dendrite attached to the base of a filiform probasisternal hair in a whole-mount preparation (Watson and Pflüger, 1984). **(C)** A scanning electron micrograph of an adult locust probasisternum showing the array of filiform hair receptors in ventral view. **(D)** A schematic drawing of the filiform hair-to-interneuron system in the locust (Pflüger et al., 1994). Abbreviations: A1, A4, first and fourth abdominal neuromeres; ant, anterior; ISI, intersegmental interneuron; M, muscle; MESO, META, meso- and meta-thoracic ganglia; Mn, Motor neuron; probas, probasisternal; proepi, proepisternal; pronot, pronotal; TAG, terminal abdominal ganglion.

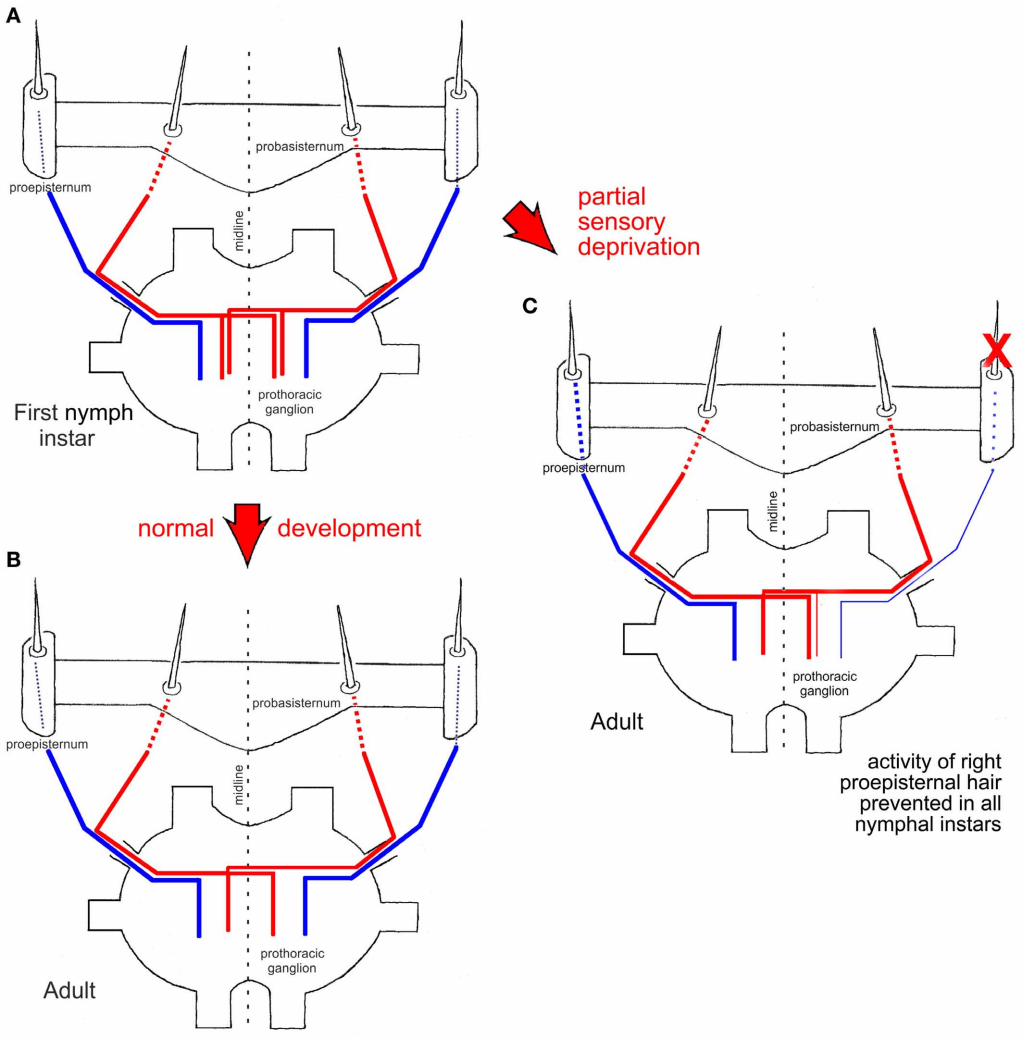

FIGURE 2 | Schematic drawings of central nervous projections of two proepisternal (blue) and two probasisternal (red) filiform hairs into the prothoracic ganglion in a first nymphal instar (A) and an adult (B) (after Pflüger et al., 1994). (C) Experimental adult animal in which the neuronal activity of one proepisternal hair had been prevented in all nymphal instars (red X). Note that the central nervous projection of the experimentally silenced proepisternal hair was present but weaker than normal [compare to **(A)**], and also note the survival of the ipsilateral central nervous projection of an adjacent probasisternal hair. The central nervous projection of a filiform hair on the probasisternum contralateral to the manipulated side remained unaffected and exhibited the normal adult pattern. Red arrows between figure parts indicate normal and experimental situations during development, respectively.

exhibited the normal elimination of ipsilateral arborizations, by contrast. Thus, an activity-dependent competition process obviously exists between the proepisternal and probasisternal hair receptors at least, in addition to the developmental hormonal process that shapes the final projections patterns of the mechanoreceptive cells of this sensory system (Pflüger et al., 1994).

THE A4I1-NEURON: INPUT AND OUTPUT CONNECTIONS

All the above mentioned wind receptors connect directly to a first-order interneuron termed A4I1 (the term signifies that the soma is located within the first unfused, that is, the fourth abdominal ganglion). This is a projection interneuron originating in the fourth abdominal ganglion with its axon ascending contralateral to the soma and terminating within the dorsal deutocerebrum. The main input, and thus the main spike initiating zone, of A4I1 is located in the prothoracic ganglion, where all the hair receptors make their direct connections. Even the small number of wind sensitive head hairs in field 1 (>5; **Figure 1A2**) project to the prothoracic ganglion and make direct connections to the A4I1 interneuron there. Again, these hairs are the first in field 1 to exist in a first nymphal instar. This morphological peculiarity of interneuron A4I1 is reflected in its firing properties: An identical burst of spikes is simultaneously sent anteriorly to the brain and posteriorly toward the fourth abdominal ganglion, thus representing a perfect corollary discharge. Corresponding to this morphology, intracellular recordings from the soma show passively invading action potentials

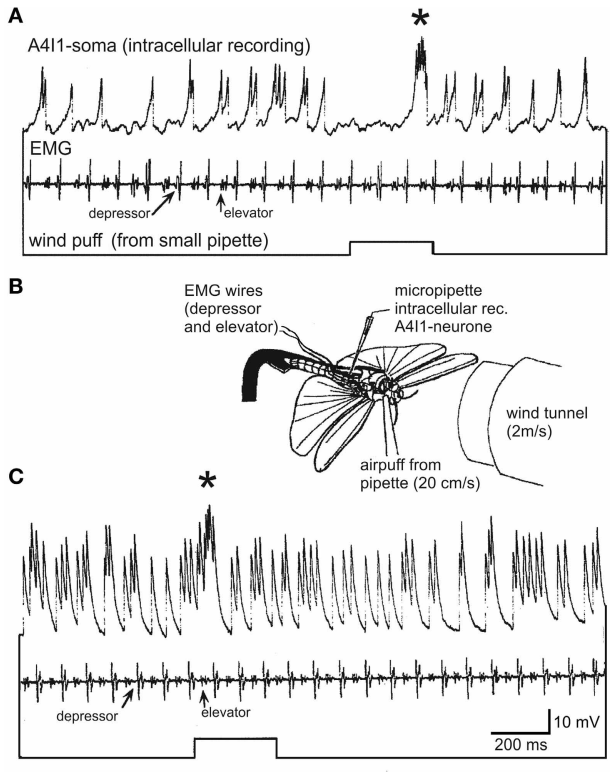

FIGURE 3 | Intracellular recording from the soma of an A4I1-interneuron (top trace), electromyogram from wing muscles (depressor and elevator, second trace), and wind puff monitor (bottom trace). The wind puff was applied to the side of the animal where the recorded A4I1 had its axon (i.e., ipsilateral to the axon, and thus contralateral to the soma). **(B)** Experimental situation (details in text). The locust was fixed to a holder and flying upside-down, and a small window was cut into the abdomen to expose the fourth abdominal ganglion which was immobilized on a small steel platform to avoid movement. 50 μm steel wires insulated except for cut end were used for electromyograms and placed into respective muscles. The locust was flying spontaneously and without head wind from the wind tunnel in **(A)**; the wind tunnel was switched on in **(C)**.

(see **Figure 3**) generated more anteriorly within the prothoracic spike initiation zone.

It was an intriguing result of these connectivity studies that the synaptic strengths of the filiform hair-to-interneuron connections were large indeed. Many of the individually identifiable filiform hairs exhibited gains of 1, or close to 1 (Pflüger and Burrows, 1990). That is, almost every spike in a hair receptor elicited a spike in interneuron A4I1.

The intriguing receptive field and high input gain of interneuron A4I1 beg the question of what the output connections of this interneuron are. Corresponding to a role in flight behavior, A4I1 makes direct connections with the motor neurons to the pleuroaxillary muscles of front and hind wings, as well as with an unidentified motor neuron to a muscle of the first abdominal segment (**Figure 1D**). The pleuroaxillary wing muscles are functional steering muscles since they control the angle of pronation and supination and, thus, adjust thrust and lift and function in all steering manoeuvres.

STRUCTURAL DYNAMICS SHAPE A4I1's RECEPTIVE FIELD?

In contrast to the number of and input from sensory receptors, the dendritic and axonal arbors of the A4I1-neuron do not dramatically change between first instars and adult locusts (Bucher and Pflüger, 2000). When the responses of the two A4I1-neurons to wind stimuli from different directions are recorded extracellularly, only quantitative changes are observed between nymphal instars and adults. In general, these changes are characterized by an increasing separation of the two neurons' receptive fields, such that only in adult animals, when flight emerges as a new behavior, the full directional sensitivity is acquired (Bucher and Pflüger, 2000).

The A4I1-neuron is not the only interneuron which receives inputs from the prothoracic wind hairs. An electrophysiological search in the prothoracic ganglion revealed additional interneurons, some with their somata within the prothoracic ganglion (Münch, 2006). Details of their connectivity and function remain as yet enigmatic, however.

HOW DOES THIS HAIR-TO-INTERNEURON SYSTEM FUNCTION IN (RESTRAINED) FLIGHT?

It is suggestive to speculate that the hair receptors of A4I1's complex receptive field monitor parameters of the air flow around the head and the frontal part of the thorax in a flying locust. Examining the air flow around a locust head with removed front legs shows that it is more or less laminar until the mesothoracic segment (Pflüger and Tautz, 1982) and that proepisternal hairs are deflected in air flow direction to a maintained position as long as the flow persists. Nothing is known about the proepisternal receptors, but if the front legs were fixed in the characteristic flight posture the air flow became turbulent, suggesting that this will also happen to the air flowing around the proepisternum.

In keeping with a role in flight behavior, output connections onto flight steering muscles suggest a role in course control. It would appear necessary to examine such hypotheses by, first, visualizing the air flow around the locust head and thorax in (tethered) flight and, second, observing possible responses to selective stimulation of the respective hair receptors in the A4I1 interneuron.

To approach the second aspect, we recorded intracellularly from the A4I1-soma and extracellularly from one pleuroaxillary muscle in a dissected locust flying upside down in front of a wind tunnel. Head wind speed was ∼2 m/s, and during fictive flight small air puffs were delivered from a cut microelectrode at ∼10-fold weaker wind speeds (20 cm/s). The opening of this microelectrode was placed opposite to the proepisternum and the space that is formed by the head and the first thoracic segment with the probasisternal hairs pointing into this space (indicated in **Figure 3B**). As shown in **Figure 3A**, the A4I1-interneuron with its axon ipsilateral (and soma contralateral) to the pipette is rhythmically excited already by the animals' own wing beat, even without any external wind stimulus (0–4 spikes per wingbeat cycle, 1.75 on average). The recorded activity represents spikes that passively invade the soma (see above) and are superimposed on depolarizations that reach the soma from the neurites. That is, the size relationships of spikes and subthreshold depolarizations are distorted. Nonetheless, A4I1 activity pattern is clearly discernible.

An air puff from the pipette causes a complex response, an initial inhibition followed by a pronounced burst (asterisk). With a head wind of 2 m/s the A4I1 neuron is excited much more strongly than during stationary flight in resting air (**Figure 3C**) (1–5 spikes per wingbeat cycle, 2.61 on average). Nonetheless, a weak turbulent air puff is clearly reflected by a burst of spikes in the recording (asterisk). No inhibition is discernible and the excitatory response occurs much earlier than in the situation without head wind. Detailed interpretation of these observations is impossible at present since the (aerodynamic) mode of stimulation of the hair sensilla is not clear, and neither is the change in the air puff stimulus brought about by the head wind. It would appear possible that with head wind present the air puff is deflected and becomes more turbulent, thus stimulating different sets of hair receptors at different strengths, which may have caused the differences in the response characteristics.

In summary, we conclude that the A4I1 hair-to-interneuron system probably monitors weak turbulences around the anterior locust body during flight. In line with this interpretation, wind alone without flight motor activity already excites A4I1 above threshold (not shown). The characteristic flight posture of the front legs may further allow the animal to direct air flow into the afore-mentioned space and thus influence or modulate the wind stimulus reaching the probasisternal, proepisternal, and pronotal hairs. Again, further study is essential here to assess the validity of these ideas. With modern laser Doppler techniques such experiments appear actually quite feasible despite difficult access to some of the hair sensilla.

KEEPING A4I1 IN A SUITABLE WORKING RANGE

The mechanosensory-to-flight motor pathway from filiform hairs to wing steering muscles via the A4I1 interneuron makes sense in a flight steering context, as does the response of the A4I1 interneuron to air puffs just presented. However, the enormous sensitivity of the filiform hair-to-interneuron connection remains intriguing. Mechanisms must exist to prevent this system from working at or close to saturation.

A few candidate mechanisms exist that may prevent the hair-to-interneuron system from reaching saturation. Among them is presynaptic gain control, described for sensory afferents from chordotonal organs (Burrows and Matheson, 1994) in walking (Wolf and Burrows, 1995), and in stridulation (Poulet, 2005; Poulet and Hedwig, 2006). Although electrophysiological study of possible presynaptic inhibition is still missing, GABAergic mechanisms are clearly in place to limit A4I1-firing (Gauglitz and Pflüger, 2001). In addition, the prothoracic neuropile is densely labeled by NO-synthase-immunoreactive profiles (Münch et al., 2010) in areas where synaptic interactions between the filiform hair receptors and the A4I1-neuron occur. And NO has been shown to effect a general decrease in the A4I1 response to a wind-puff (Münch et al., 2010).

CONCLUSIONS

Not just in holometabolous insects but in hemimetabolous insects as well, sensory and motor neurons may exhibit remarkable structural and functional dynamics, dependent on the respective developmental context. In addition to hormonal regulation, which provides a developmentally programmed time frame, activity-dependent mechanisms adjust sensory receptors to individual characters. This is evident when sensory receptors are ablated and synaptic rearrangement including structural dynamics occurs, even in adult insects. For example, interneurons may connect to sensory receptors they would never receive input from under normal conditions (Murphey, 1986; Brodfuehrer and Hoy, 1988; Kanou et al., 2004). The locust filiform hair-to-interneuron system involving A4I1 is suitable for such studies, particularly with regard to its well-known output connections, by comparison to other systems.

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Glia-related circadian plasticity in the visual system of Diptera

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The circadian changes in morphology of the first visual neuropil or lamina of Diptera represent an example of the neuronal plasticity controlled by the circadian clock (circadian plasticity). It is observed in terminals of the compound eye photoreceptor cells, the peripheral oscillators expressing the clock genes. However, it has been found also in their postsynaptic partners, the L1 and L2 monopolar cells, in which the activity of the clock genes have not yet been detected. The circadian input that the L1 and L2 receive seems to originate not only from the retina photoreceptors and from the circadian pacemaker neurons located in the brain, but also from the glial cells that express the clock genes and thus contain circadian oscillators. This paper summarizes the morphological and biochemical rhythms in glia of the optic lobe, shows how they contribute to circadian plasticity, and discusses how glial clocks may modulate circadian rhythms in the lamina.

Keywords: circadian plasticity, glia, visual system, *Drosophila, Musca*

INTRODUCTION

The visual system of Diptera exhibits daily rhythmic changes in morphology and physiology (Pyza and Górska-Andrzejak, 2008; Pyza, 2010). To maintain synchrony with environmental cycles, this rhythmicity is entrained by an external cycle of day and night (LD), but it is generated predominantly by the endogenous pacemakers, the circadian clocks, that produce oscillations with a period of approximately 24 h. Due to timing signals from the clocks, the phenomenon of rhythmic changes persists also in the condition of constant darkness (DD), representing an example of a particular type of plasticity, the circadian plasticity (Frenkel and Ceriani, 2011; Mehnert and Cantera, 2011).

CIRCADIAN PLASTICITY OF NEURONS IN THE VISUAL SYSTEM OF DIPTERA

Studies on the housefly, *Musca domestica* and the fruit fly, *Drosophila melanogaster* have shown that in the visual system of Diptera (**Figure 1**), the circadian plasticity manifests itself both in the retina of the large compound eye (**Figure 1A**) (Chen et al., 1992) and in the first visual neuropil beneath the compound eye, the lamina (**Figure 1B**) (Pyza and Górska-Andrzejak, 2008; Pyza, 2010). In the retina, the circadian clock regulates the process of phototransduction, the sensitivity of photoreceptors to light, and their adaptation to changing light conditions (Giebultowicz, 2000; Pyza, 2010). In the underlying lamina, the circadian control is even more pronounced (Pyza and Meinertzhagen, 1997). In the so called cartridges—the synaptic units of lamina neuropil (**Figure 2**)—both the terminals of photoreceptors (R1–R6) and the axons of their most conspicuous postsynaptic partners (the L1 and L2 interneurons, cf. **Figure 2A**) exhibit robust structural rhythms (Pyza and Meinertzhagen, 1995, 1997, 1999; Górska-Andrzejak et al., 2005; Barth et al., 2010). It has been shown that in the fruit fly the volume of photoreceptor terminals changes in a circadian manner (Barth et al., 2010), whereas in the housefly the endogenous reorganization of organelles within R1–R6 terminals is maintained under circadian modulation (Pyza and Meinertzhagen, 1997). In *Musca*, the number of screening pigment granules and the number of inter-receptor invaginations from neighboring terminals show circadian changes (Pyza and Meinertzhagen, 1997). The number of synaptic contacts between R1 and R6 terminals and axons of L1, L2 monopolar cells (the tetrad synapses) also undergoes certain changes over the course of 24 h, but this modulation was found to be rather weak and not of circadian origin (Pyza and Meinertzhagen, 1993). In case of *Drosophila*, changes in the number of tetrad presynaptic ribbons have been reported as circadian by Barth et al. (2010). Nevertheless, additional studies that could provide more quantitative insight into the origin of tetrads daily fluctuations would be helpful in clarifying this issue.

Since photoreceptors of the compound eye belong to the peripheral oscillators expressing the core genes of the circadian clock - *period* (*per*) and *timeless* (*tim*) (Siwicki et al., 1988; Zerr et al., 1990; Cheng and Hardin, 1998)—their circadian plasticity might be expected. On the other hand, until now the expression of clock genes have not been detected in their postsynaptic partners—the L1 and L2 monopolar cells. Yet, they display robust circadian rhythms (Pyza and Meinertzhagen, 1993, 1995, 1999; Górska-Andrzejak et al., 2005; Weber et al., 2009). The axons of L1 and L2 in the lamina of three fly species: *Musca domestica*, *Drosophila melanogaster,* and *Calliphora vicina* (the blow fly) change their size in a circadian manner, though in a different pattern for each species. These patterns are correlated with the increase in fly's daily locomotor activity (Pyza and Meinertzhagen, 1993, 1999; Pyza and Cymborowski, 2001). In the housefly, also the number of so called feedback synapses in L2 axons shows circadian fluctuations (Pyza and Meinertzhagen, 1993). The feedback synapses form back onto the R1–R6 terminals in the proximal lamina (Meinertzhagen and Sorra, 2001;

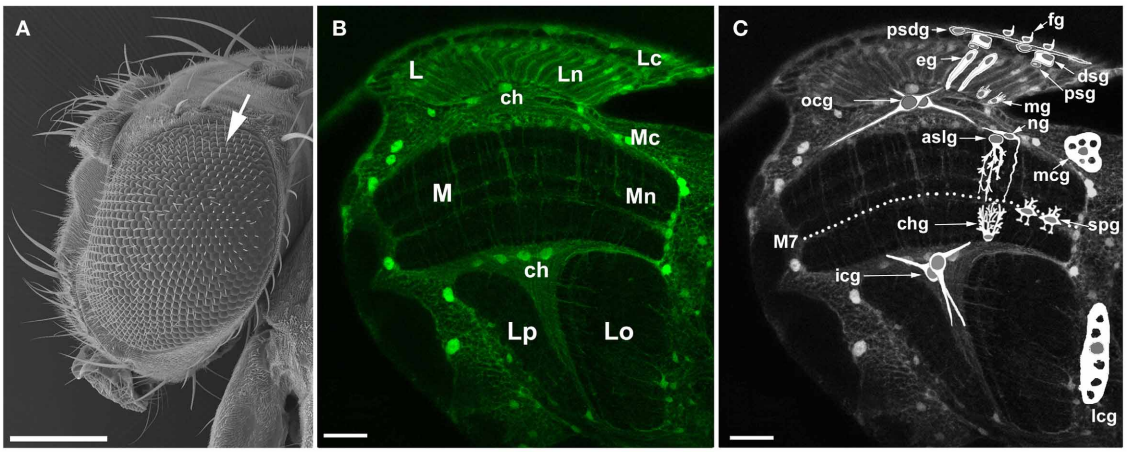

FIGURE 1 | The visual system of the fruit fly, *Drosophila melanogaster*. **(A)** Scanning electron micrograph of a head and a large compound eye. The eye is composed of approximately 800 hexagonal units called facets or ommatidia (arrow). The ommatidial array of photoreceptors in the retina receives photic and visual information, transduces it into receptor action potentials and transmits to underlying optic lobe. Scale bar: 200 μm. **(B)** Confocal image of the optic lobe of transgenic flies Repo-Gal4 × UAS-S65T-GFP, in horizontal section. Targeted expression of Green Fluorescence Protein (GFP) to glial cells reveals the general morphology of the optic lobe. There are three synaptic regions (neuropils) beneath the retina of the compound eye: the lamina (L), the medulla (M), and the lobula that in Diptera consists of the lobula (Lo) and the lobula plate (Lp). Lc, lamina cortex; Ln, lamina neuropil; Mc, medulla cortex; Mn, medulla neuropil; ch, chiasm. Scale bar: 20 μm. **(C)** Schematic representation of so far identified types of glia (based on Edwards et al., 2012) revealing their general morphology and relative locations in the optic lobe: fg, fenestrated glia; psdg, pseudocartridge glia; dsg, distal satellite glia; psg, proximal satellite glia; eg, epithelial glia; mg, marginal glia; mcg, medulla cortex glia; aslg, astrocyte-like glia of the distal medulla neuropil; ng, another type of the distal medulla neuropil glia; spg, serpentine glia; chg, chandelier glia; ocg, outer chiasm glia (giant and small ocg); icg, inner chiasm glia; lcg, lobula cortex glia; M7, the serpentine layer. Certain types of glia (eg, aslg, and/or ng, mcg, lcg, ocg, icg) can be also discern in the tissue visible in the background being marked by GFP. Scale bar: 20 μm.

Górska-Andrzejak et al., 2013). Considering that the hyperpolarization of L2 by current injection does not alter the photoreceptors response (Laughlin and Osorio, 1989), the functional significance of transmission at these synapses is not entirely clear and the precise meaning of its circadian nature is difficult to explain. However, the network of interneurons, with L2 among them, does modulate the speed and amplitude of photoreceptors response: when the signal transfer from photoreceptors to interneurons is low, e.g., in dim light condition, the synaptic feedback increases to boost photoreceptors output (Zheng et al., 2006). Taking this into account, one can expect that the L2 monopolar cell at least partly shapes the photoreceptor's response by negative feedback loop, even though it forms only a few feedback synapses (Meinertzhagen and Sorra, 2001). This influence of L2 on photoreceptors output appears to be controlled by the circadian clock because their number increases at the beginning of the night both in LD and DD conditions (Pyza and Meinertzhagen, 1993).

The circadian remodeling of synaptic contacts in Diptera visual system manifests itself also in daily fluctuations in the level of expression of the presynaptic protein Bruchpilot (BRP) (Górska-Andrzejak et al., 2013), which has been shown to localize to the platform of photoreceptor synaptic ribbons (Górska-Andrzejak et al., 2009a; Hamanaka and Meinertzhagen, 2010) and to play the role of the master organizer of the synaptic active zone in *Drosophila* neuromuscular junction (Fouquet et al., 2009). When examined in the distal part of *Drosophila* lamina in LD (12 h of light and 12 h of darkness—12:12), the level of BRP increases twice: at the beginning of the day and at the beginning of the night (Górska-Andrzejak et al., 2013). Interestingly, while the evening peak depends on the circadian input in an essential way, the morning peak of BRP abundance does not appear to be controlled by the circadian clock depending rather on light influence and phototransduction pathway in the retina photoreceptors. Significant alterations in the level of BRP over the course of a 24 h day (Górska-Andrzejak et al., 2013) imply light-dependent and clock-dependent control over the lamina synapses, and ultimately over the neuronal transmission of photic information in the lamina.

Studies on transgenic lines of *Drosophila* (21D-Gal4 × UAS-GFP), in which the morphology of L2 was labeled by cytoplasmic or membranous GFP reporter protein revealed, that in addition to the circadian changes in axon size and the number of feedback synapses, the entire structure of this cell (**Figure 3**) undergoes daily remodeling (Górska-Andrzejak et al., 2005; Weber et al., 2009). The size of cell nuclei and, more importantly, the size of a dendritic tree in the lamina also oscillate during the 24 h day, with the highest amplitude of changes at the beginning of the day (Górska-Andrzejak et al., 2005; Weber et al., 2009). Interestingly, L2 dendrites that are postsynaptic to photoreceptors are the longest at the beginning of the day (Weber et al., 2009), which coincides with the increase of the number of tetrad synapses in photoreceptor terminals (shown in *Musca*) and with the level of BRP in the lamina (shown in *Drosophila*) (Pyza and Meinertzhagen, 1993; Górska-Andrzejak et al., 2013). This coincidence strongly suggests the possibility of correlation, which

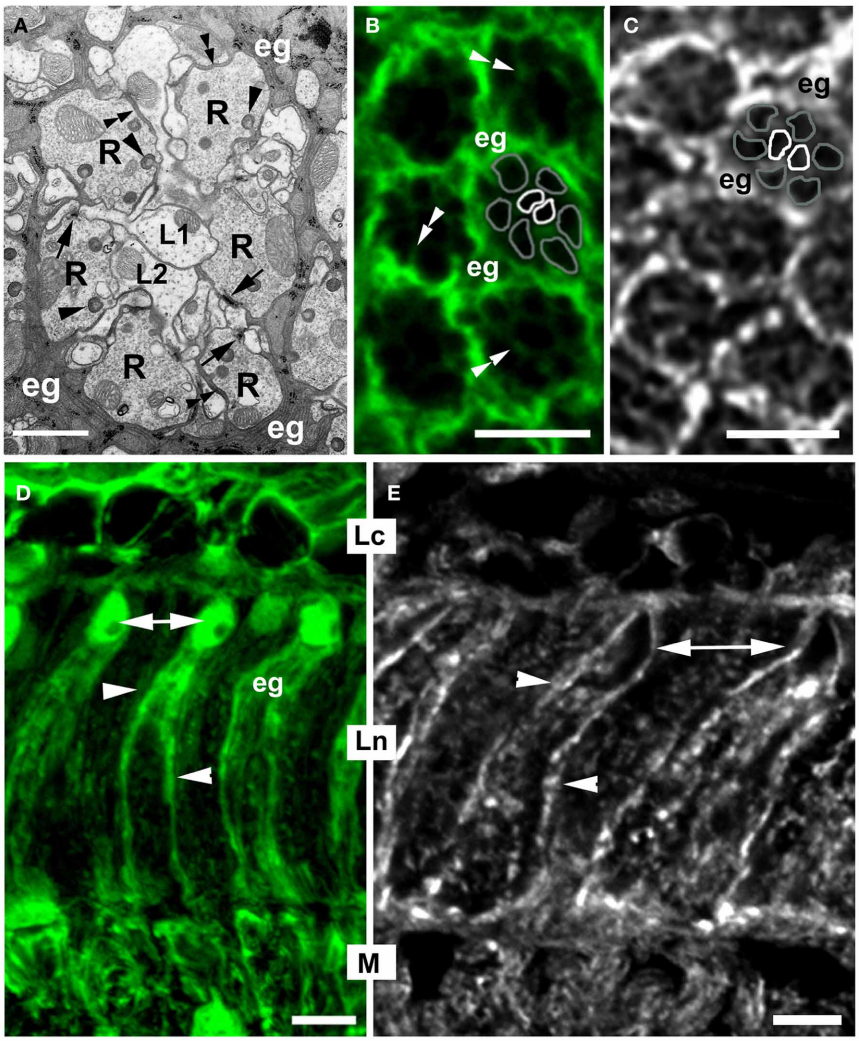

FIGURE 2 | The morphology of the cartridge in cross- (A–C), and longitudinal (D,E) section of *Drosophila melanogaster* first visual neuropil or lamina. (A) EM microphotograph of a single cartridge. The cylindrical terminals of six photoreceptors (R) surround axons of their main target cells, the L1 and L2 lamina interneurons, the so called large monopolar cells (LMCs). Apart from these, each cartridge hosts also smaller processes (not marked) of two more photoreceptors, R7 and R8, and three other monopolar cells, L3–L5, as well as profiles of amacrine cells, whose cell bodies are located in the second visual neuropil or medulla, and tangential neurons with cell bodies in other parts of the brain. Each cartridge is enwrapped by three cells of epithelial glia (eg) that send small processes into the cartridge (double arrowheads) and deep, dynamic invaginations with a spherical head, called capitate projections (arrowheads) into photoreceptor terminals. R-terminals contain profiles of presynaptic ribbons (T-bars) of the tetrad synapses (arrows), the most abundant type of synapses in the lamina. They constitute approximately 59% of the total number of all synapses of a cartridge (Meinertzhagen and Sorra, 2001). These synapses transmit photic and visual information received by ommatidia toward the brain. Scale bar: 1 μm. **(B)** Confocal image of the cartridges of Repo-Gal4 × UAS-S65T-GFP transgenic flies. GFP labels the epithelial glia (eg) surrounding cartridges. There are also visible thin processes (double arrowheads) invading the cartridge. One of the cartridges contains schematic representation of its main components, namely the terminals of R1–R6 photoreceptors (gray outline) and the axons of L1, L2 monopolar cells (white outline). Scale bar: 5 μm. **(C)** The pattern of α5 Mab (Hybridoma) immunolabeling of Na^+/K^+-ATPase α-subunit in the cartridges of lamina. The strongest signal comes from the epithelial glia (eg). As in **(B)**, one of the cartridges contains schematic representation of R1–R6 terminals (gray outline) and the axons of L1 and L2 (white outline). Scale bar: 5 μm. **(D)** GFP fluorescence in the lamina glial cells of Repo-Gal4 × UAS-S65T-GFP transgenic flies. Epithelial glia (eg) are localized in the synaptic part of the lamina (Ln). Their cell bodies with nuclei (arrows) are located in the distal part of the neuropil, whereas their processes (arrowheads) reach the proximal part of the neuropil. Lc, lamina cortex; M, medulla. Scale bar: 5 μm. **(E)** Immunoreactivity to the α-subunit of the Na^+/K^+-ATPase in the longitudinal section of lamina. Similarly as in the lamina cross section **(C)**, the strongest fluorescence is visible in the membrane of cell bodies (arrows) and processes (arrowheads) of the epithelial glia. Scale bar: 5 μm.

however should be confirmed in a direct experiment. Presumably, there are also corresponding changes in L1, which partners L2 (**Figures 2A**, **3A**), although analogous studies of L1 morphology have been so far hindered by the lack of a driver for L1.

The precise mechanism underlying generation of circadian rhythmicity in the monopolar cells of the lamina remains largely unknown. However, if L1 and L2 are not hosting the circadian oscillators themselves, than they must receive the circadian input

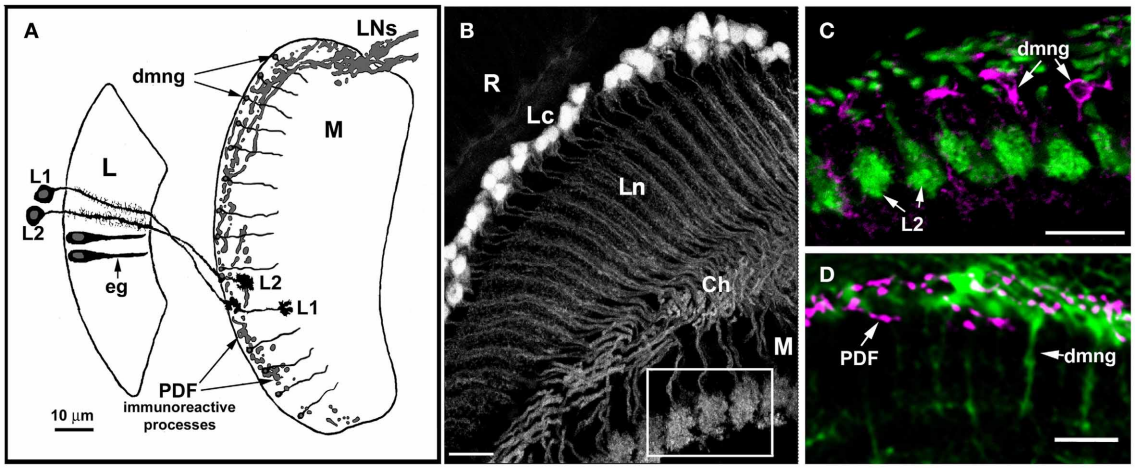

FIGURE 3 | Anatomical relationships between glial cells (eg, epithelial glial cells of the lamina, dmng, distal medulla neuropil glia) and their putative targets, the L1 and L2 monopolar cells, as well as terminals of some clock neurons (LNs). (A) Diagram showing the relative locations of L1 and L2 in the lamina and distal medulla, the terminals of PDF immunoreactive lateral neurons (LNs), lamina neuropil epithelial glia (eg), and distal medulla neuropil glia (dmng). L, lamina; M, medulla. **(B)** Morphology of the L2 monopolar cell in the optic lobe of 21D-GAL4 × UAS-S65T-GFP transgenic flies. Cell bodies of L2 are distributed beneath the retina (R), in the region of the lamina cortex (Lc); the axons and dendrites of these cells are in the lamina neuropil (Ln), and their terminals in the medulla (M). Ch, external chiasm. Scale bar: 10 μm. **(C,D)** The region of the medulla seen as an insert in **(B)**. **(C)** GFP labeled terminals of L2 and distal medulla neuropil glia (dmng) showing Ebony-like immunoreactivity. Scale bar: 10 μm. **(D)** The distal medulla neuropil glia (dmng) of Repo-Gal4 × UAS-S65T-GFP transgenic flies and terminals of PDH-immunoreactive LNs (PDF) labeled with anti-PDF antibody. The PDF-positive LNs send projections into the region where the terminals of L2 and Ebony-expressing dmng are located. Scale bar: 10 μm.

as the target cells located downstream in the circadian pathway. The increasing amount of data shows that the circadian input to the lamina may originate not only from the retina photoreceptors (Cheng and Hardin, 1998) and the circadian pacemaker neurons of the brain (Bałys and Pyza, 2001; Damulewicz and Pyza, 2011), but also from glial cells of the optic lobe (Pyza and Górska-Andrzejak, 2004; Górska-Andrzejak et al., 2009b, 2013).

Glial cells of the *Drosophila* optic lobe (**Figure 1C**) belong to surface, cortex, neuropil, and tract glia (Edwards and Meinertzhagen, 2010). They differ by their location, morphology, gene expression, and function (recently described in Edwards and Meinertzhagen, 2010; Hartenstein, 2011; Edwards et al., 2012). Underneath the retina of the large compound eye, at the surface of the lamina, there are two distinct subtypes of the surface glia: the fenestrated glia (fg; perineurial layer) and the pseudocartridge glia (psdg; subperineurial layer). Below them, the lamina is populated by two types of the cortex glia (also called "satellite," when located in the visual system) and two types of the neuropil glia. The so called distal (dsg) and proximal (psg) satellite glia ensheath the cell bodies of monopolar neurons and the photoreceptor axon bundles in the lamina cortex, whereas the epithelial (eg) and marginal (mg) glia reside in the lamina synaptic neuropil, where they ensheath neuronal processes (**Figure 1C**). The epithelial glia has been recently reported to be the lamina counterpart of the so called astrocyte-like neuropil glia, even though its columnar morphology (**Figure 2D**) does not resemble the astrocyte-like one (Edwards et al., 2012). The cortex glia and the neuropil glia are also present in the second visual neuropil or medulla. Medulla cortex glia (mcg) typically for this type of glia form a mesh of processes that encapsulate the neighboring neuronal cell bodies, whereas the medulla neuropil glia extend their processes deep into the synaptic neuropil (**Figure 1C**). Distinct types of the medulla neuropil glia have been recently described based on differences in morphology and localization (Edwards et al., 2012). There are at least two morphologically different types of the neuropil glia in the distal part of the medulla neuropil (dmng), with the first one representing the astrocyte-like glia (aslg) and the second having different kind of morphology (ng) (**Figure 1C**). Apart from the distal part, the astrocyte-like glia reside in other parts of this neuropil as well; the serpentine glia (spg) in the posterior margin of M7 (the serpentine layer) and the chandelier glia (chg) at the base of the medulla (Edwards et al., 2012). Like the first two, the third visual neuropil—the lobula complex is populated by cortex and neuropil glia as well (Eule et al., 1995; Tix et al., 1997). The astrocyte-like glia have also been reported among the lobula neuropil glia, although not characterized further (Edwards et al., 2012). The axon tracts projecting between the visual neuropils are wrapped by the tract glia (**Figure 1C**) (Edwards and Meinertzhagen, 2010). These are glia of the outer (ocg) and the inner (icg) optic chiasmata (between the lamina and the medulla and between the medulla and the lobula, respectively) (**Figure 1C**). When the outer chiasm is populated by the giant and the small ocg, the inner chiasm hosts only the giant glia. Each of the above mentioned types/subtypes of glia not only has a unique morphology and function, but also contacts a unique group of optic lobe neurons (Edwards et al., 2012).

GLIAL OSCILLATORS

Glial cells of Diptera seem capable of taking part in the circadian modulation of neuronal circuitry of the lamina, first and foremost because they themselves contain circadian oscillators (Zeng et al., 1994; Jackson, 2011). It has been known for more than two

decades that certain types of glial cells express *per,* the core gene of the mechanism for keeping circadian time, and that the abundance of its product (PER) in glia, like in the neurons, fluctuates in a circadian manner (Siwicki et al., 1988; Zerr et al., 1990; Ewer et al., 1992). The fruit fly glia express also another clock gene: *tim* (Suh and Jackson, 2007). In the transcriptional negative feedback loop of *Drosophila* molecular clock (the core loop), PER and TIM proteins form heterodimers to enter the nucleus and inhibit the transcription of their own genes by repressing transcription activator composed of Clock (CLK) and Cycle (CYC). This generates oscillations in the level of their expression in the cell (Hardin, 2005). If not all, than at least those glial cells that contain the PER-based molecular oscillator are well equipped to function as rhythm generators and modulators (Zeng et al., 1994). In *Drosophila*, the glial cells of the visual system also belong to the *per* expressing glia (Siwicki et al., 1988; Ewer et al., 1992).

GLIA DERIVED CIRCADIAN MODULATION OF THE LAMINA NEURONAL CIRCUITRY

INVOLVEMENT OF THE EPITHELIAL GLIA IN THE CIRCADIAN VOLUME CHANGES OF L1, L2 AXONS

In the lamina synaptic neuropil of *Musca domestica*, the spontaneous cyclical changes that follow a circadian rhythm have been detected not only in the morphology of the L1 and L2 monopolar cells, but also in morphology of the epithelial glia (**Figure 2D**) (Pyza and Górska-Andrzejak, 2004). In *Musca* and *Drosophila* three of such cells surround each synaptic cartridge (Boschek, 1971; Meinertzhagen and O'Neil, 1991) sending their projections toward its inside (**Figures 2A,B**). Squeezing between much bigger profiles of R1–R6 and L1, L2, the glial projections localize themselves in close proximity to the neuronal profiles and their synaptic connections. In case of photoreceptors, the epithelial glia make characteristic close invaginating appositions into their terminals (**Figure 2A**): the so called capitate projections (Edwards and Meinertzhagen, 2010). The precise function of these intimate connections between photoreceptors and glial cells is still quite obscure. They are known, however, to be involved in vesicle endocytosis (Fabian-Fine et al., 2003).

The epithelial glia have been found to undergo daily alterations in cell morphology (Pyza and Górska-Andrzejak, 2004). It is not much of a surprise in view of close association of the lamina epithelial glia with the photoreceptor terminals and axons of L1, L2 monopolar cells that display circadian changes. Epithelial glial cells of *Musca* expand at night and shrink during the day, exactly in antiphase to daily changes of L1 and L2 neurons (Pyza and Górska-Andrzejak, 2004). (Axons of L1 and L2 swell during the day, and shrink by night). This suggests some kind of a compensatory relationship between L1, L2 and surrounding epithelial glia. Possibly, glial cells that contain the PER-based oscillator (epithelial glia express *per*; Siwicki et al., 1988) as the part of the circadian system provide some degree of control over the volume changes in L1, L2. The possibility of such control is corroborated by data showing that daily changes in glia morphology influence the rhythmic structural changes of L1 and L2 axons (Pyza and Górska-Andrzejak, 2004). Disrupting the metabolism of glia by injecting glial metabolic toxins, e.g., fluorocitrate or iodoacetate (Kitano et al., 2003) affects the neuronal rhythm by increasing its amplitude (Pyza and Górska-Andrzejak, 2004). Alterations in the neuronal rhythm occur also when the communication between the lamina cells via the gap junction channels is disrupted (Pyza and Górska-Andrzejak, 2004). Most of the gap junctions in the lamina are formed between glial cells (Saint Marie et al., 1984). Therefore, the uncoupling effect of the application of gap junction closing agent, such as octanol (Pappas et al., 1996), is observed predominantly on the "glial part" of the lamina. The contribution of gap junctions formed between neurons, however, cannot be entirely ruled out. In the condition of decreased coupling between glial cells, the amplitude of both glial and neuronal rhythm was observed to be noticeably smaller (Pyza and Górska-Andrzejak, 2004). In fact, in case of L2, the differences between the volume of day-axons and night-axons were no longer significant. Such results indicate, that the epithelial glia of the lamina modulate the circadian rhythmicity of the target neurons L1 and L2. Interestingly, the decreasing cell volume effect of octanol in neurons was stronger during the day, whereas in glia it was stronger during the night (Pyza and Górska-Andrzejak, 2004).

CIRCADIAN EXPRESSION OF Na^+/K^+-ATPase IN THE VISUAL SYSTEM

In *Drosophila melanogaster*, the circadian changes in the volume of lamina monopolar cells are accompanied by daily changes in the expression of the Na^+/K^+-ATPase, the sodium pump (Górska-Andrzejak et al., 2009b). The sodium pump is the major pump that regulates the ionic homeostasis in many different cell types (Lingrel et al., 1990). Therefore, its engagement in the mechanism of neuronal and glial cells volume regulation, including diurnal modulation, might have been expected. In *Drosophila* optic lobe, the expression of Na^+/K^+-ATPase were indeed observed to be under the circadian control at both mRNA and protein level (Górska-Andrzejak et al., 2009b). However, of the α and β subunits of the sodium pump (responsible for the catalytic and regulatory functions, respectively), only the first one showed a robust, *per*-dependent circadian rhythm of concentration (Górska-Andrzejak et al., 2009b).

The relative amount of the α subunit protein was at the highest level at the beginning of the night and at the lowest level at the end of the night, as revealed by the immunolabeling intensity for this subunit (Górska-Andrzejak et al., 2009b). Such pattern of daily changes of the sodium pump coincides with the above-mentioned pattern of oscillations in L1 and L2 axon sizes (Pyza and Meinertzhagen, 1999). It is also interesting to note that the immunospecific labeling for the α subunit was particularly strong in the first and the second optic neuropil. It was detected in most cells of the optic lobe, but in the lamina the particularly intense signal was visible in the epithelial glia (**Figures 2C,E**), especially in their membranes, where the protein is active (**Figure 2E**) (Górska-Andrzejak et al., 2009b). It is thus possible that predominantly the glial oscillators contribute to the rhythm of the sodium pump. In view of these observations, L1 and L2 appear as likely targets for circadian modulation driven by epithelial glia, which at certain times of the day might provide such ion buffering capacity that is required by axons of highly active neurons.

It was also reported that the Na^+/K^+-ATPase may have a pump-independent function. It is required for cell junctions formation (Genova and Fehon, 2003; Paul et al., 2007). Therefore, it

is possible that Na^+/K^+-ATPase is involved in neuron—glia communication. If this were the case, the observed circadian changes in its expression in the optic lobe of *Drosophila* would additionally mean the existence of the circadian control over neuron-glia communication.

In case of the β-subunit of the Na^+/K^+-ATPase (encoded by the *Nervana 2*), the results were less conclusive (Górska-Andrzejak et al., 2009b). The fluorescence intensity of GFP reporter revealed only small, statistically insignificant differences between day and night. However, it might have been partly due to limitations imposed by the variant of the GFP reporter (S65T-GFP) driven by *Nrv2* (Sun et al., 1999) used in these studies. Interestingly, the GFP signal was strong in the glial cells of the medulla, especially in the distal medulla neuropil glia (dmng) (**Figures 1C**, **3**). Their cell bodies are located between the cortex and the neuropil of the medulla, whereas their long processes penetrate the neuropil. It is difficult to determine to which type of the distal medulla glia (aslg or ng, perhaps both) the *Nerv2*-expressing glia belong to. Still, their processes run close to the terminals of L2 and L1 (**Figures 3A,C**), and close to dense varicose arborization of the terminals of the circadian clock neurons—the lateral neurons (LNs) (**Figures 3A,D**). LNs terminals are immunoreactive to pigment dispersing factor (PDF), a transmitter in the circadian system (Saifullah and Tomioka, 2003) and synchronizer of different clock neurons in the system (Lin et al., 2004). Apart from close localization to LNs terminals that release the PDF, these glial cells express *per*. Because of such characteristics, these type of glia may have great capacity to modulate the circadian rhythmicity in neurons, such as L1 or L2. However, further studies are needed to establish their role in this process.

GLIAL INFLUENCE ON CIRCADIAN PATTERN OF BRP EXPRESSION

Recent findings also suggest that glial cells influence the above described pattern of circadian expression of the presynaptic BRP in distal lamina of *D. melanogaster*. It appears that the bimodal pattern of daily changes in the amount of BRP in the cartridges of lamina depends not only on circadian oscillators in photoreceptors and the clock neurons of the brain but also on the glial oscillators (Górska-Andrzejak et al., 2013).

In per^0 mutants, in which the circadian system is disrupted, both peaks in BRP abundance disappear and the rhythm is abolished. However, when only the glial circadian oscillator is disrupted (by dominant negative form of the Cycle protein, CycΔ#103) or the expression of *per* is silenced exclusively in the glia (by *per-RNAi* construct), the daily changes in BRP accumulation in the lamina cartridges still take place, but their pattern is considerably altered (Górska-Andrzejak et al., 2013). Glial cells as the component of the circadian system apparently modulate the pattern of circadian expression of BRP, and therefore they appear to have an influence on daily changes in neurotransmission in the lamina of Diptera.

CIRCADIAN EXPRESSION OF GLIA-SPECIFIC PROTEIN *EBONY*

Studies on the expression and function of *ebony* gene in *Drosophila*, revealed another interesting information about "circadian capacity" of glial cells: they can drive the behavioral circadian rhythms (Suh and Jackson, 2007). *ebony*—the key player in this process—encodes a protein of N-β-alanyl-biogenic amine synthase activity (Ebony), an enzyme that conjugates β-alanine to biogenic amines, such as dopamine and serotonin (Hovemann et al., 1998; Richardt et al., 2003). The fact that *ebony* mutants display arrhythmic locomotor activity (Newby and Jackson, 1991) and that in the head of a non-mutant fly the *ebony* mRNA shows robust circadian cycling (Ueda et al., 2002) strongly suggest that this protein modulates circadian behavior.

The most important, however, is that in *Drosophila* head Ebony localizes exclusively to glial cells. By discovering this fact and showing that glial expression of Ebony rescues the phenotype of *ebony* mutants, Suh and Jackson (2007) demonstrated a connection between glial cells and the circadian control of a locomotor behavior of the fruit fly. They reported Ebony as the first identified glial factor required for behavioral rhythmicity. They also proposed a model in which Ebony participates in the circadian control of dopaminergic functions and circadian activity rhythms (Suh and Jackson, 2007).

In the visual system of *Drosophila*, similarly as in the whole brain, Ebony protein localizes exclusively to glial cells. As Suh and Jackson (2007) reported, the majority of Ebony-positive glia in fly brain belongs to neuropil glia. In the visual system, the Ebony-positive glia have been identified as the lamina epithelial glia (eg), the neuropil glia of distal medulla (dmng), (**Figures 1C**, **3C**) (Richardt et al., 2003) and recently also the serpentine glia (**Figure 1C**) (Edwards et al., 2012). These are incidentally the same glial cells that express PER protein (Siwicki et al., 1988; Zerr et al., 1990; Ewer et al., 1992), display circadian changes in morphology and influence circadian rhythms of L1 and L2 (eg) (Pyza and Górska-Andrzejak, 2004), express the Na^+/K^+-ATPase in a circadian manner (eg and dmng), and are localized in the area of PDF release from the terminals of LNs (dmng) (Górska-Andrzejak et al., 2009b). Ebony-positive glia of the visual system are therefore definitely involved in its circadian modulation.

In the visual system of *Drosophila* Ebony is required for the high level of daytime activity. It conjugates β-alanine to histamine forming β-alanyl-histamine, or carcinine (Borycz et al., 2002; Richardt et al., 2003; Ziegler et al., 2012). By expressing Ebony, epithelial glial cells take part in the recycling of the photoreceptors neurotransmitter—histamine, and appear to regulate circadian photoreception (extensively reviewed in Edwards and Meinertzhagen, 2010 and Jackson, 2011).

CONCLUSIONS

The visual system of Diptera is well established as a model for the circadian rhythms studies. The photoreceptor cells of the large compound eye of *Drosophila* express clock genes and are by themselves the peripheral oscillators. The optic lobe of *Drosophila*, on the other hand, contains the circadian system that consists of two components: the circadian clock neurons—the LNs and the PER-expressing glial cells. The main directions of research have focused on different groups of clock neurons, their circuitry, and ways of transmitting the circadian information—circadian neurotransmitters and their receptors. In comparison with the clock neurons, the glial cells have received considerably less attention.

Incidentally, certain types of glial cells may actually be called "glial clocks" (Jackson, 2011). They contain PER-based circadian

oscillators (Siwicki et al., 1988; Zerr et al., 1990; Ewer et al., 1992; Zeng et al., 1994; Jackson, 2011) and appear to be the integral part of the circadian system as partners for the clock neurons rather than only supportive cells (Pyza and Górska-Andrzejak, 2004; Suh and Jackson, 2007; Górska-Andrzejak et al., 2009b, 2013; Jackson, 2011; Ng et al., 2011).

Out of the four main glial types described in the brain and visual system of *Drosophila* (Edwards and Meinertzhagen, 2010), only certain types of the neuropil glia have been shown to take part in circadian modulation of neuronal circuitry (and behavior) (Jackson, 2011). They extend their membranes around axons or axon bundles (Edwards and Meinertzhagen, 2010) and form intimate connections with neurons in the synaptic part (neuropil) of the nervous structure. In the optic lobe of *Drosophila melanogaster* and *Musca domestica* these are the epithelial glial cells of the lamina neuropil and the neuropil glia of the medulla. Both of them modulate the circadian rhythmicity of L1 and L2 interneurons, which are the output cells in the circadian system of the optic lobe (**Figure 3**) (Pyza and Górska-Andrzejak, 2004; Górska-Andrzejak et al., 2009b). The precise function of the medulla neuropil glia in modulating L1, L2 rhythms, however, has been described mainly through indirect evidence and requires further studies.

Obviously, the clock neurons (LNs) play a crucial role in *Drosophila* pacemaker, and in the circadian plasticity of the visual system. Breaking the connection between the housefly's optic lobe and the rest of the brain abolishes the shrinking and swelling rhythm of L1, L2 axons changes (Bałys and Pyza, 2001). The recent studies revealed also the direct input from LNs to the lamina, which uses an ITP-like peptide as a neurotransmitter (Damulewicz and Pyza, 2011).

One might wonder then why would clock neurons need glia to convey the circadian information if they have means to directly send the signal to the target cells, like L1, L2? A good answer may have been given in the studies on glia involvement in circadian axon changes in L1 and L2 (Pyza and Górska-Andrzejak, 2004). Pathologic glia with disrupted metabolism are no longer able to counteract the neuronal size changes, which results in excessive increase in the amplitude of the rhythm (Pyza and Górska-Andrzejak, 2004). This suggest not only that L1 and L2 are the proximal targets for glial control of their circadian plasticity but also that the function of these glial cells is to stabilize the proper level of daily changes. To reach these goals the glial cells appear to work as a synchronized network. Diminishing the normal level of inter-glial/glial-neuronal synchronization by closing gap junction channels between them results in flattening of the neuronal rhythm (Pyza and Górska-Andrzejak, 2004). Localization and strong expression of PER in the medulla neuropil glia, which contact the PDF immunoreactive terminals of LNs (**Figure 3C**) implies that these cells may receive instructive circadian signals from clock neurons. The glial clocks, however, are also capable of modulating the clock neurons: the recent studies on *Drosophila* neuropil glia of astrocyte type showed that the glial cells can physiologically modulate the circadian clock neurons (Ng et al., 2011). It has also been shown that the activity of the two ubiquitous transcription factor families that are involved in numerous cellular processes: cAMP response element-binding protein (CREB) and nuclear factor kappa-B (NF-κB), remains under circadian regulation both in neurons and in glia (Tanenhaus et al., 2012). In view of the observations presented in this short review one may conclude that glial clocks and neuronal clocks are partners.

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Photoperiodic plasticity in circadian clock neurons in insects

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Since Bünning's observation of circadian rhythms and photoperiodism in the runner bean *Phaseolus multiflorus* in 1936, many studies have shown that photoperiodism is based on the circadian clock system. In insects, involvement of circadian clock genes or neurons has been recently shown in the photoperiodic control of developmental arrests, diapause. Photoperiod sets peaks of *period* (*per*) or *timeless* (*tim*) mRNA abundance at lights-off in *Sarcophaga crassipalpis*, *Chymomyza costata* and *Protophormia terraenovae*. Abundance of *per* and *Clock* mRNA changes by photoperiod in *Pyrrhocoris apterus*. Subcellular Per distribution in circadian clock neurons changes with photoperiod in *P. terraenovae*. Although photoperiodism is not known in *Leucophaea maderae*, under longer day length, more stomata and longer commissural fibers of circadian clock neurons have been found. These plastic changes in the circadian clock neurons could be an important constituent for photoperiodic clock mechanisms to integrate repetitive photoperiodic information and produce different outputs based on day length.

Keywords: photoperiodism, circadian clock neurons, plasticity, Per, s-LNv, pigment-dispersing factor

INTRODUCTION

Photoperiod is an important cue for organisms to obtain information on calendar time from the environment. Plants and animals respond to the regular changes in day length to coordinate their development and physiology to these seasonal changes. This is called photoperiodism. This review focuses on plasticity or changes in gene expression and neural morphology that might be involved in photoperiodic mechanisms in insects. The physiological mechanisms underlying photoperiodism comprise three components: photoperiodic photoreceptors, photoperiodic clocks, and endocrine systems. Photoperiodic information is processed by neural elements responsible for the photoperiodic clock. The outputs from the photoperiodic clock affect the endocrine systems in which the release of humoral factors controls developmental programs (Saunders, 2002). The photoperiodic clock has been considered to consist of a two-step process: one step being the time-measurement system, which measures the day or night portion of a day and the other is the photoperiodic counter. In the latter step, photoperiodic information is successively received during the sensitive period and it then accumulates to an internal threshold. Once this threshold is passed, the endocrine events controlling development are triggered. It is quite interesting how the brain integrates repetitive photoperiodic information as well as whether any neural plasticity is involved in the photoperiodic clock. However, very few studies have addressed these issues.

Bünning (1936) had first proposed that an endogenous circadian oscillator is involved in the time-measurement system for photoperiodic responses. His original idea was based on the observations of leaf movements in the runner bean *Phaseolus multiflorus*. He found two distinct phases comprising a circadian cycle: one is called "photophil," a light-requiring phase (upward movement of the leaf), and the other, "scotophil," a darkness-requiring phase (downward movement of the leaf). *P. multiflorus* exhibits photoperiodism and its flowering is promoted under short-day conditions. Bünning found that longer period of exposure to light during scotophil phase in *P. multiflorus* delayed the flowering time compared to shorter period of exposure to light (Bünning, 1936). He reached the idea that photoperiod is measured by referring to the phases of the circadian clock that are entrained to environmental light and dark cycles. Since then, many pieces of experimental evidence have supported the circadian clock-based time-measurement, but only recently the involvement of circadian clock genes or neurons has been shown in case of insects.

In the temperate region insects grow and reproduce in favorable seasons whereas they enter diapause, developmental arrest, in unfavorable seasons. Diapause is controlled by photoperiod in many insects. In-depth molecular studies of the circadian clock mechanism have been successfully undertaken in *Drosophila melanogaster* (Peschel and Helfrich-Förster, 2011), but the temperature is the main determinant of dormancy, and photoperiod has small effects on it (Emerson et al., 2009; Hahn and Denlinger, 2011). Therefore, the involvement of circadian clock mechanisms in photoperiodism has been analyzed in different species. Some clear results were obtained showing the necessity of circadian clock genes in the photoperiodic control of diapause by using mutant analysis or RNA interference technique. A circadian clock gene *timeless* (*tim*) has been shown to be an important gene for the photoperiodic response of a drosophilid fly *Chymomyza costata*, *period* (*per*) for the cricket *Modicogryllus siamensis*, and both *per* and *Cycle* (*Cyc*) for the

bean bug *Riptortus pedestris* (Pavelka et al., 2003; Sakamoto et al., 2009; Ikeno et al., 2010). Ablation experiments have suggested that circadian clock neurons driving locomotor activity rhythms are prerequisite to photoperiodism in the blow fly *Protophormia terraenovae* (Shiga and Numata, 2009). However, it still has to be unveiled how these circadian clock genes or neurons play roles in photoperiodism.

It is unknown how photoperiodic information is encoded in the neural system. Photoperiod is composed of a fixed period (t h) of light and 24-t h of darkness. Discrimination of light from darkness can be simple, but measuring t or 24-t h and discriminating long days from short days are more complex processes. Encoding photoperiodic information requires a time-coding system. This can be handled by two ways: one refers to the circadian clock phases (described by Bünning) and the other uses a non-oscillating hourglass, which is set in motion in each cycle at light-off and measure night length by referring to critical night length (Putterill et al., 2010). It is quite possible that coding information in a rather long time period needs neural plasticity. As no idea is so far available for hourglass mechanisms by neurons, here I discuss aspects of plasticity in gene expression, subcellular clock protein location and neural morphology that are observed in circadian clock neurons under different photoperiods.

PHOTOPERIODIC CHANGES IN CLOCK GENE EXPRESSION

Studies on the molecular machinery underlying the circadian clock in *D. melanogaster* show that many clock genes and proteins interacting in at least three interdependent feedback loops are involved in rhythm generation (Peschel and Helfrich-Förster, 2011). Circadian fluctuations in the abundance of *per* and *tim* mRNA and their proteins are an important element in the feedback loops. One important characteristic of the circadian oscillator is entrainment to the environmental cycles. The molecular basis of entrainment mechanisms to light-dark cycles (photoperiodic cycles) has been understood partially. During photoperiodic cycles, Cryptochrome (Cry) is activated by "lights-on" leading to Timeless (Tim) degradation, and because of the lack of Tim, Per is vulnerable to phosphorylation and subsequently degraded, thus starting a new cycle (Lin et al., 2001). The Cry signal is terminated by "lights-off" leading to nighttime accumulation of Per and Tim (Qiu and Hardin, 1996). Thus, Cry photoreception in the clock neurons is a key feature of photoperiodic entrainment. Considering their characteristics under different photoperiods, the temporal accumulation patterns of the circadian clock mRNA and proteins can be expected to undergo changes. In fact, the mRNA of *per* and *tim* of the head sample reach their peaks in abundance 4 h after "light-off" during photoperiods of 16-h light and 8-h darkness (LD, 16:8), LD 12:12, and LD 8:16, and oscillation uses "lights-off" as a phase reference point (Qiu and Hardin, 1996). Setting peaks of *per* or *tim* mRNA levels at "lights-off" have also been observed in the head of the flesh fly *Sarcophaga crassipalpis*, the whole central nervous system of *C. costata*, and the brain of *P. terraenovae* (Goto and Denlinger, 2002; Stehlík et al., 2008; Muguruma et al., 2010). Other types of responses of circadian clock gene expression to photoperiods have also been observed. In the head of the linden bug *Pyrrhocoris apterus*, clear oscillation has not been observed, but *per* mRNA abundance was 10-fold higher and *Clock* mRNA was 2-fold higher under long-day conditions than under short-day conditions (Syrová et al., 2003). The circadian clock gene expression responds to photoperiods, although the responses vary among species.

PHOTOPERIODIC CHANGES IN THE SUBCELLULAR LOCATION OF CIRCADIAN CLOCK PROTEINS

In the current model of the circadian clock system in *D. melanogaster*, the very center of the clock mechanism is characterized by the activation of *per* and *tim*. The evening-time increase in mRNA leads to the accumulation of Per and Tim in the cytoplasm. Tim and Per enter the nucleus alone or as a heterodimer allowing Per-associated double-time (Dbt) kinase to co-enter the nucleus. This transport is mediated via Per phosphorylation. Inside the nucleus, the Per-Dbt-Tim complex accumulates and binds to Clock (Clk)/Cycle (Cyc) dimers via Per-Clk interaction, causing the hyperphosphorylation of Clk to prevent Clk/Cyc dimers from binding to E-Box sequences in the promoter region of many circadian clock-regulated genes, including *per* and *tim*. This inhibits the transcription of *tim* and *per* toward "lights-on." Per's inhibition of its own transcription generates a negative feedback loop. Cyclic expression of Per and Tim protein and mRNA have been observed previously (Peschel and Helfrich-Förster, 2011).

Using transgenic lines or immunocytochemistry, the subcellular distribution of the circadian clock proteins has been examined. In *D. melanogaster*, about 150 neurons express circadian clock genes in the brain. These neurons are classified into seven groups and designated according to their anatomical locations. By using markers recognizing molecules involved in circadian clock systems, each group of clock neurons was further divided into several subgroups (Kaneko and Hall, 2000; Peschel and Helfrich-Förster, 2011). Among them, the small type of ventral lateral neurons (s-LN_vs) was considered the main circadian oscillator for behavioral rhythm under constant darkness. The effects of day length on the temporal profiles of Per and Tim accumulation in the nuclei or cytoplasm have been studied in s-LN_vs. The temporal patterns of the subcellular distribution of clock proteins change according to the photoperiod: both Tim and Per were almost absent in the cell shortly after "lights-off," and at "lights-on" protein accumulation was obvious in the nuclei, especially in Per during both long-day and short-day conditions (Shafer et al., 2004). This means that nuclear localization timing is affected by photoperiod in *D. melanogaster*. In addition, in *P. terraenovae*, a similar distribution of Per-immunoreactive cells to *D. melanogaster* has been revealed (Shiga and Numata, 2009). The subcellular distribution of Per-immunoreactivity was compared between the long-day and short-day conditions in four types of circadian clock cells, namely, s-LN_vs, large type of ventral lateral neurons (l-LN_vs), dorsal lateral neurons (LN_ds), and medial dorsal neurons (DN_ms) (Muguruma et al., 2010). In all cell types, Per-immunoreactivity in the nucleus was the highest 12 h after "lights-off" and lowest 12 h after "lights-on" irrespective of

photoperiod. This results in a clear differences in Per distribution at "lights-on" between the photoperiod: under short days, Per is mainly localized in the nucleus, whereas Per-immunopositive nuclei were less in number under long-day conditions at "lights-on." Per nuclear translocation seems to entrain to photoperiod. The temporal patterns of Per staining under short-day conditions slightly differed among the cell types of *P. terraenovae*. In l-LN_vs and s-LN_vs, Per remains in the nucleus for a longer period of time during photophase than in LN_ds and DN_ms. It is known that in *D. melanogaster*, Per is stabilized by Tim, but without protection by Tim during photophase Per is phosphorylated and degraded. The degradation process of Per might be slow in the s-LN_vs and l-LN_vs, compared with LN_ds and DN_ms in *P. terraenovae*.

In *D. melanogaster*, two circadian oscillators, namely, morning and evening oscillators, have been functionally proposed to drive locomotor rhythms. Stoleru et al. (2007) suggested differential circadian photo-entrainment features present between the "evening cells" of LN_ds, DN1s, and DN2s, and "morning cells" of sLN_vs. These features with inter-oscillator communication may underlie circadian adjustment to the changes in photoperiod. GFP expression driven by clock gene promoters has very well-revealed the fiber distribution of circadian clock neurons (Helfrich-Förster, 2003). The clock neurons form a network with their fine terminals in dorsal protocerebrum, which houses fibers from the neurosecretory cells in the brain. This circadian neural network must encode day-length information, and it may submit the output signals to the dorsal protocerebrum, where neurosecretory cells control diapause or reproduction (Shiga and Numata, 2000).

CIRCADIAN AND PHOTOPERIODIC CHANGES IN FIBER DISTRIBUTION IN CLOCK NEURONS

Fiber distribution of the circadian clock neurons also shows daily plasticity in *D. melanogaster* (Fernández et al., 2008). By using a membrane-bound version of GFP under the *pigment-dispersing factor* (*pdf*)-specific promoter, the morphological plasticity of the terminal fibers of s-LN_vs was examined. The s-LN_vs have somata at the ventral and anterior bases of the medulla in the optic lobe, and extend their axonal fibers posterior-dorsally to the protocerebrum. In the dorsal protocerebrum s-LN_vs make trajectory shifts and open varicose fibers two-dimensionally by the somata of DN1s and DN2s. As the s-LN_v branches in the dorsal protocerebrum are labeled by a presynaptic marker, these branches are considered terminal sites. The rhythmic alterations in the structure were found to contain higher complexity in fiber distribution during the day, and significantly lower complexity during the night. These oscillations continue under both light-dark cycles of 24 h and constant dark conditions. These structural changes disappear in the clock-less mutants of *D. melanogaster*. These morphological changes could be a mechanism for transmitting clock information in a time-dependent manner, and may indicate a change in the terminal fibers of s-LN_vs connecting to different targets at different times during the day (Fernández et al., 2008).

PDF-immunoreactive neurons in the cockroach *Leucophaea maderae* show morphological plasticity when reared under different photoperiods (Wei and Stengl, 2011). In the cockroach, the circadian clock controlling the locomotor activity rhythms is located at the accessory medulla with associated PDF-immunoreactive neurons. According to cell size and location, PDF-immunoreactive neurons are classified into five types. One type called medium-sized aPDFMes obviously responds to changes in day length. With the increase in day length, the amount of PDF-immunoreactive somata has increased and longer commissural fibers have been found. Because visual stimulation or monocular deprivation during development causes changes in optic lobe volume, this difference could be simply caused by difference in visual experience (Barth et al., 1997). However, considering the fact that photoperiod seems to differentially affect PDF-ir neurons, this photoperiodic change in medium-sized aPDFMes suggests some role in photoperiodic adjustment in the clock mechanism. Because *L. maderae* is a tropical species as is *D. melanogaster*, this does not show phenotypic plasticity like diapause induction by photoperiod. It would be interesting to investigate morphological plasticity in the circadian clock neurons in insects showing clear photoperiodism.

During the examination of physiological mechanisms underlying photoperiodism, molecular and neural activities are easily taken into account. However, dynamic changes of neural morphology may be one approach to investigate neural coding of photoperiodic information. In the Japanese quail *Coturnix japonica*, which shows clear photoperiodism controlling gonadal maturation, morphological changes are known to occur in the terminal sites of the gonadotropin-releasing hormonal neurons under different photoperiods (Yamamura et al., 2004). Immuno-electron microscopy revealed that gonadotropin-releasing hormonal nerve terminals are in close proximity to the pericapillary space for secreting neurohormone into portal blood under long days, but that their terminals are encased by glial cells under short-day conditions. In future, it might prove interesting to examine the differences in morphological plasticity in the circadian clock neurons between long-day and short-day conditions in insects showing clear photoperiodism.

CONCLUSION

Photoperiodic changes have been reported in the expression patterns of the circadian clock genes, in the subcellular distribution of clock proteins, and in fiber distribution or the number of clock neurons. These plastic changes in the circadian clock neurons could tell photoperiodic information to some unknown neuronal networks, where day counting must occur up to a certain threshold. When the number of days passes the threshold, neurosecretory systems are excited or suppressed to control development and diapause. Because circadian output fibers are intermingled, and neurosecretory cells in the pars intercerebralis and pars lateralis are located, the dorsal protocerebrum could furnish important neural networks for photoperiodic clock system.

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Quantification of dendritic and axonal growth after injury to the auditory system of the adult cricket *Gryllus bimaculatus*

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Dendrite and axon growth and branching during development are regulated by a complex set of intracellular and external signals. However, the cues that maintain or influence adult neuronal morphology are less well understood. Injury and deafferentation tend to have negative effects on adult nervous systems. An interesting example of injury-induced compensatory growth is seen in the cricket, *Gryllus bimaculatus*. After unilateral loss of an ear in the adult cricket, auditory neurons within the central nervous system (CNS) sprout to compensate for the injury. Specifically, after being deafferented, ascending neurons (AN-1 and AN-2) send dendrites across the midline of the prothoracic ganglion where they receive input from auditory afferents that project through the contralateral auditory nerve (N5). Deafferentation also triggers contralateral N5 axonal growth. In this study, we quantified AN dendritic and N5 axonal growth at 30 h, as well as at 3, 5, 7, 14, and 20 days after deafferentation in adult crickets. Significant differences in the rates of dendritic growth between males and females were noted. In females, dendritic growth rates were non-linear; a rapid burst of dendritic extension in the first few days was followed by a plateau reached at 3 days after deafferentation. In males, however, dendritic growth rates were linear, with dendrites growing steadily over time and reaching lengths, on average, twice as long as in females. On the other hand, rates of N5 axonal growth showed no significant sexual dimorphism and were linear. Within each animal, the growth rates of dendrites and axons were not correlated, indicating that independent factors likely influence dendritic and axonal growth in response to injury in this system. Our findings provide a basis for future study of the cellular features that allow differing dendrite and axon growth patterns as well as sexually dimorphic dendritic growth in response to deafferentation.

Keywords: sexual dimorphism, anatomical plasticity, midline guidance

INTRODUCTION

Anatomical plasticity in the adult central nervous system (CNS) is presumed to be important for successful recovery from serious neuronal injuries. However, only a handful of examples of such plasticity in the CNS in either adult vertebrates or invertebrates have been described, mainly in a few different species or in particular injury scenarios (Murphey et al., 1975; Bulloch and Ridgway, 1989; Büschges et al., 1992; Wolf and Büschges, 1997; Krüger et al., 2011b; Tavosanis, 2012). One of the more striking, positive, compensatory responses to injury has been described in the auditory systems of several species of field crickets (Hoy et al., 1985; Schildberger et al., 1986; Brodfuehrer and Hoy, 1988). Crickets have evolved a mechanism to compensate for injury to the auditory system, a sensory system crucial to cricket survival and reproduction, and because this compensatory response is unusual for any species in the animal kingdom, it is worthy of in-depth study.

The compensatory response in the cricket is complex. It involves both sprouting of deafferented dendrites and of intact, contralateral axons as a direct and indirect result of deafferentation, respectively, as well as novel synapse formation and functional recovery. Unlike the cricket, many denervation experiments in embryonic and developing animals typically result in post-synaptic neuronal decline and death (Parks, 1979; Trune, 1982a,b; Nordeen et al., 1983; Born and Rubel, 1985) while denervation in mature animals minimally affects post-synaptic neurons (Born and Rubel, 1985; Hashisaki and Rubel, 1989; Moore, 1990). The general conclusions derived from these studies are that pre-synaptic input is necessary for growth and stabilization of their post-synaptic partners during development, but once neuronal systems mature in the adult, many classes of neurons reach a stable state and do not need active input to maintain their structure (Tavosanis, 2012).

Denervation-induced withdrawal and death does not occur in all systems, and intriguing exceptions have been identified in both vertebrates and invertebrates. For example, deprivation of sensory input in adult rodents, via whisker removal or forepaw denervation, leads to indirect denervation of cortical neurons and

has been shown to induce dendritic reorganization (Hickmott and Steen, 2005; Tailby et al., 2005). However, additional studies have failed to detect large-scale reorganization of cortical dendrites after visual deprivation (Hofer et al., 2008) or even after direct retinal damage (Keck et al., 2008) in the adult. Physical injury to neurons via axotomy can lead to varying amounts of dendritic and axonal growth as well as shifts in dendritic arbor organization, as is seen in mouse superior cervical ganglion cells (Yawo, 1987), lamprey central neurons (Hall and Cohen, 1983), feline neck motor neuron (Rose et al., 2001), cricket terminal ganglion interneurons (Chiba et al., 1988; Chiba and Murphey, 1991), and in the cricket and locust auditory systems (Pallas and Hoy, 1986; Lakes and Kalmring, 1991; Krüger et al., 2011b). Deafferentation-induced elevator motor neuron sprouting in the metathoracic ganglion of locusts helps these animals recover proper wing patterns after unilateral tegula removal (Büschges et al., 1992; Wolf and Büschges, 1997). Within the cricket, deafferentation-induced compensatory dendritic responses have been demonstrated in both the terminal ganglion and the prothoracic ganglion (Murphey et al., 1975; Murphey and Levine, 1980; Hoy et al., 1985; Pallas and Hoy, 1986; Schildberger et al., 1986; Brodfuehrer and Hoy, 1988; Schmitz, 1989; Kanou et al., 2004; Horch et al., 2011; Krüger et al., 2011a,b). The deafferentation-induced compensation in the terminal ganglion consists mainly of changes in the strength of existing synapses (Murphey and Levine, 1980), though some dendritic sprouting has been noted (Murphey et al., 1975). The deafferentation-induced functional recovery in the auditory system of the cricket, on the other hand, is a result of extensive post-synaptic sprouting and reorganization as well as sprouting from intact, contralateral axons in both juveniles and adults (Schmitz, 1989; Horch et al., 2011). It is notable that functional recovery in the auditory system appears to require that axons and dendrites grow across the midline, a landmark that typically serves as an inhibitory boundary (Pallas and Hoy, 1986; Brodfuehrer and Hoy, 1988; Schmitz, 1989).

Adult crickets detect sound waves with tympanal membranes and associated auditory organs that are located on the tibial section of the cricket's forelegs. Auditory information is conveyed to the prothoracic ganglion in the CNS via nerve 5 (N5), and the auditory receptor axons end in a claw-shaped arbor within the medioventral association center (mVAC). Auditory interneurons located in the prothoracic ganglion are arranged in bilaterally symmetric pairs on either side of the midline and receive their primary auditory information from the ipsilateral N5. Individual, identified auditory interneurons respond specifically to either the calls of male conspecific crickets [Ascending Neuron-1 (AN-1) responds best to 5 kHz], or the ultrasound pulses of predatory bats [Ascending Neuron-2 (AN-2) responds best to 15 kHz and higher]. These responses are shaped by the inhibitory input received by the ANs that is thought to come from the paired omega neurons (Hardt and Watson, 1994). N5 tonotopy is arranged such that afferents carrying low frequency information to AN-1 are tightly clustered in the most anterior medial portion of N5 where they meet densely clustered AN-1 dendrites. N5 afferents carrying ultrasound information are somewhat more diffuse and primarily lie along the medial edge of the anterior portion of the N5. AN-2 dendrites in turn span the medial edge of N5. Low and ultrasound frequency variscosities overlap in some areas of N5 (Wohlers and Huber, 1985; Imaizumi and Pollack, 2005). N5 carries additional axons into the prothoracic ganglion that originate in additional sensory organs. Many of these axons arborize more laterally, though a small group arborizes in the peripheral region of the mVAC (Nishino and Sakai, 1997; Nishino, 2000). Upon removal of one foreleg, which results in the removal of the auditory organ and the deafferentation of the auditory interneurons on that side, these auditory interneuron dendrites in both larvae and adults sprout across the midline, forming functional synapses with the contralateral auditory nerve (Hoy et al., 1985; Schildberger et al., 1986; Brodfuehrer and Hoy, 1988). The majority of crickets deafferented as adults regain responsiveness to auditory stimulation in the deafferented interneurons between 4 and 6 days after ear removal, and physiological recordings demonstrate a partial recovery of cell-type-specific auditory responses (Brodfuehrer and Hoy, 1988).

Few studies have quantified anatomical characteristics of the compensatory growth after deafferentation. Previously, our group quantified bilateral adult auditory anatomical changes induced by chronic deafferentation throughout larval development (Horch et al., 2011). However, the quantification of the anatomical changes induced by deafferentation in the adult has been explored in only a few studies (Brodfuehrer and Hoy, 1988; Schmitz, 1989). The Schmitz study (1989) describes deafferented AN-2 dendrites approaching and crossing the midline by about 7 days and reaching their maximum extent by 27 days. This study also demonstrates that the number of contralateral N5 axons projecting across the midline is significantly greater in deafferents than in controls but that the distance the N5 axons extend over the midline is not significantly different between controls and deafferented animals. These results identify general growth trends of AN-2 and N5 after deafferentation in females (Schmitz, 1989), but fine time-scale measurements were not made and male data were not collected. Thus, it is currently unclear if male and female growth responses differ. Furthermore, the precise timeline of the growth responses in three dimensions has never been examined.

In the present study we focused on quantifying three-dimensional changes in N5 axons and in the dendrites of ANs over time. Modern neuronal tracers and three-dimensional reconstructions of confocal images were used to trace and measure AN dendrites and N5 axons. Control *Gryllus bimaculatus* N5 and AN morphology were compared to that seen after 30 h as well as after 3, 5, 7, 14, and 20 days of deafferentation. Growth patterns were statistically analyzed, and here we report the most detailed account thus far of deafferentation-induced compensatory growth over time. Most intriguingly, we demonstrate that female AN dendrites grow at significantly different rates than male dendrites and differently from axons in either males or females.

METHODS

SUBJECTS

Gryllus bimaculatus (originally supplied by Ron Hoy, Cornell University) were raised in a 12:12 light:dark cycle at 28°C in 40–60% humidity. They were fed cat chow and water *ad libitum*. For neuroanatomical studies both male and female adult crickets,

no more than 1 week past adult molt, were used. After cooling, one prothoracic leg was removed proximal to the tympanal membrane. Right and left leg deafferents were prepared in roughly equal numbers, and since no differences were found, all left/right data were combined. Control crickets and those deafferented for 30 h, 3, 5, 7, 14, or 20 days were grouped by deafferentation length and housed under the same conditions as above.

BACKFILLING CRICKET ASCENDING NEURONS AND CONTRALATERAL NERVE 5

Backfills were performed as described by Horch et al. (2011). Briefly, backfills were performed "*in situ*" in cooled, immobilized crickets. Neck connective axons and nerve 5 axons were separately submerged in Vaseline wells containing one of two different neuronal tracers. A small number of axons, including the AN axons, were filled with 4% unconjugated biocytin (Sigma-Aldrich, St. Louis, MO, USA) in 50 mM $NaHCO_3$ (American Bioanalytical, Natick, MA, USA). N5 axons, those contralateral to the amputation in deafferented animals, were backfilled with 1 mg/μL biocytin conjugated to Alexa Fluor 594 (Invitrogen, Carlsbad CA, USA) in cricket saline (140 mM NaCl, 5 mM KCl, 7 mM CaCl2-2H2O, 1 mM MgCl2-6H2O, 5 mM TES, 4 mM NaHCO3, 5 mM trehalose, pH 7.3). Dye was allowed to be transported at 4°C for approximately 16–20 h, after which tissue was fixed and biocytin was visualized using 1:400 streptavidin conjugated to Alexa Fluro 488 (Invitrogen, Carlsbad, CA), as described by Horch et al. (2011). Tissue was rinsed, dehydrated through an ethanol series, and mounted, on a slide with coverslip spacers, ventral side up in methyl salicylate.

CONFOCAL MICROSCOPY

Backfilled ganglia were treated with streptavidin and imaged with a Zeiss LSM510 META laser scanning confocal microscope (Thornwood NY, USA). Three-dimensional images of ANs and N5 were taken with a Plan-NEOFLUAR 10X/0,30 and the Plan-NEOFLUAR 40X/1,3 objectives using the 488 nm and 543 nm lasers. For greater magnification, images were visualized under the 40× objective with a digital 2× zoom. To capture the location of the midline, the 633 nm laser was used to collect corresponding 40× images of the autofluorescent cells at the midline.

AN AND N5 ANATOMICAL QUANTIFICATION

AN and N5 growth across the midline were analyzed and quantified "blindly" in coded images using Volocity High Performance 3D Imaging Software (PerkinElmer, Waltham MA, USA). Autofluorescent glial cells collected with 633 nm laser were a reliable marker of the midline, which was represented by a single line running through the glial cells (shown in blue, **Figures 2A,B**). This line was then transferred, based on coordinate locations, to images of ANs and N5 (**Figure 2C**).

Using Volocity to scroll through sequential planes, axonal and dendritic branches were traced by hand in three dimensions. AN and N5 processes were quantified by the perpendicular extent of the point furthest from the midline (perpendicular extent), by the longest dendrite, and by skeletal length (the summed length of all the processes; **Figure 2D**). The volume of axons that project outside the typical medial ventral association center (mVAC) region was also measured. The upper region of the N5 "claw-shaped" arborization has a distinct posterior edge in control crickets that was approximated by a straight line for analysis. The volume of axons that projected across this line or across the mid-line was measured using Volocity (**Figure 5**).

DATA ANALYSIS

The tracing measurements and volumetric data sets were analyzed using the non-parametric Spearman rank correlation to determine whether there were significant trends in the data. When there were significant trends in the data, data were fit with linear and non-linear fitting techniques. Standard linear regression techniques were used for linear fits:

$$y = mx + b \tag{1}$$

where y is the variable of interest, x is time in days, m is the slope of the line and b is the intercept; m and b are fitted terms. Non-linear fits were done by using a standard von Bertalanffy growth function, an equation commonly used to describe growth rates over time:

$$y = l_\infty - (l_\infty - l_0)\ e^{-k\,x} \tag{2}$$

where y is the variable of interest, x is time, l_∞ is the ultimate value of y, l_0 is the value of y at time = 0 and k is a rate of change; l_∞, l_0, and k are fitted terms.

The Akaike information criterion corrected model (AICc) was used to compare whether to select the non-linear or linear fit to the data. If the probability given by AICc that the linear model was correct was greater than 50% then the linear model was selected; if the probability that the linear model was correct was less than 40% then the non-linear model was selected; otherwise the preferred model was considered "undetermined."

For all data the end-point of measured variables for males versus females was statistically compared; t-tests were used when the data met the assumptions (normality and equality of variances) of the parametric t-test, otherwise a non-parametric Kruskal–Wallis test was used for the end-point comparisons. In addition, an ANCOVA was used to compare genders if neither of the genders were better fit by the non-linear fit and if at least one of the genders had a significantly non-zero slope when fit with linear regression analysis. A non-parametric Kruskal–Wallis test on all the data was used to compare genders when there were no linear trends in the data. Within-animal AN skeletal length was compared with N5 skeletal length, N5 extraneuropillar growth, N5 extraneuropillar growth at 5 and 7 days, and Male N5 extraneuropillar growth using Spearman rank correlation to determine whether there were significant correlations in the growth of AN and N5.

RESULTS

Backfills using neuronal tracers allowed us to visualize AN dendrites (for simplicity, only AN-2 is shown in green) and N5 axons (red) in the same prothoracic ganglia in control adult crickets (**Figure 1**). Our backfills typically result in robust fills of AN-2, but sometimes include weak or incomplete backfills of AN-1

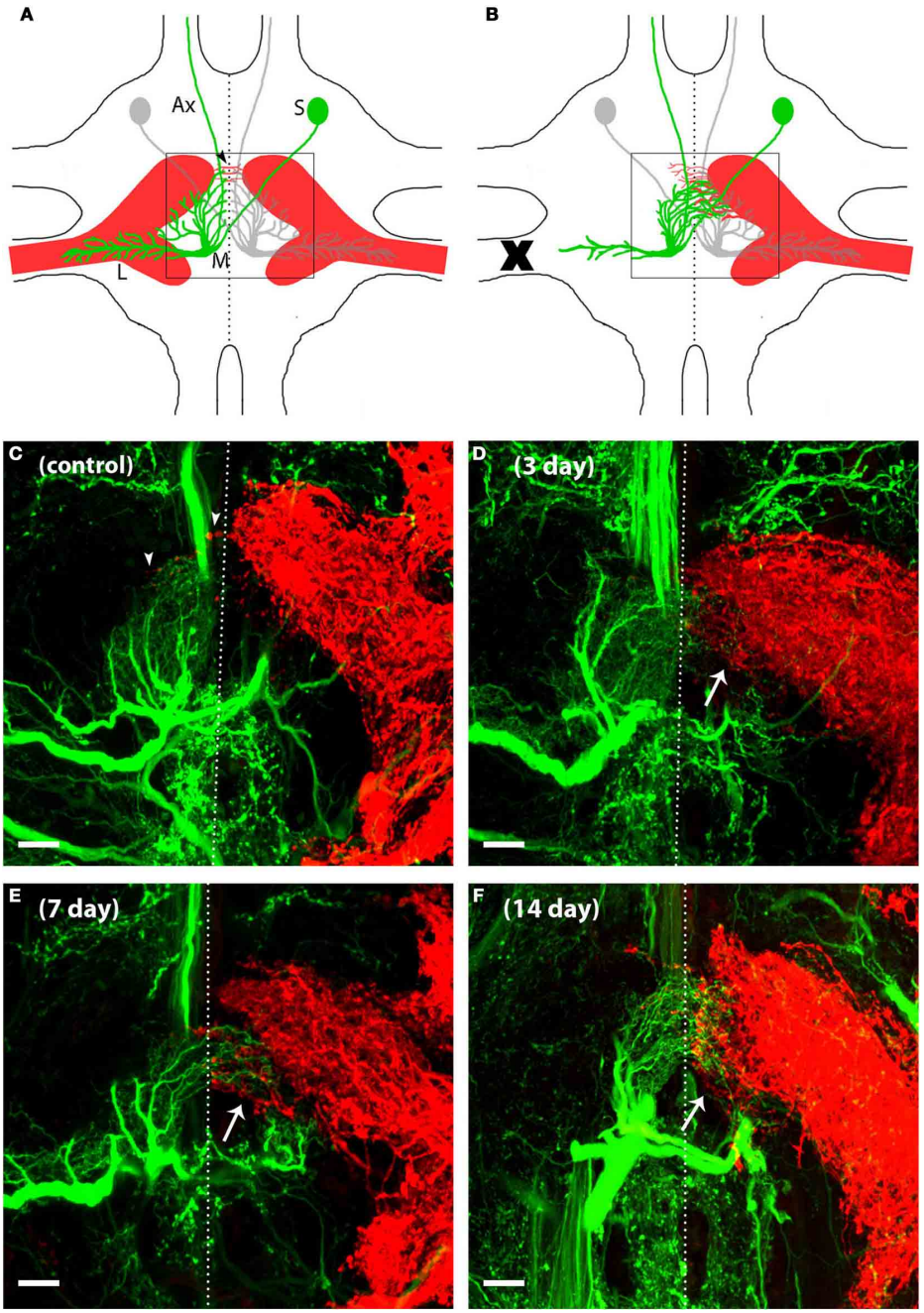

FIGURE 1 | AN dendrites and N5 axons reorganized their neuronal structure to grow toward one another after deafferentation in adult crickets. N5 (red) is composed in part of axons that carry auditory information from the tympanal organ on the cricket's forelegs to the prothoracic ganglion where a variety of neurons, including AN-2 (green), receive the auditory information and send this information to the brain. **(A)** The schematic of a prothoracic ganglion in a control animal shows that AN-2 as well as N5 are bilaterally symmetric. The N5 axonal terminal structure resembles a "claw" shape. AN-2 dendrites are arranged in an L shape, with medial dendrites (M) synapsing with the upper portion of the ipsilateral N5 claw and lateral dendrites (L) extending into the N5 axonal track. Postsynaptic information is relayed to the brain via the AN-2 axon (Ax) that extends through the anterior end of the prothoracic ganglion. The AN-2 soma (S) is located on the contralateral side of the midline from its dendrites. In control animals a few N5 axons may extend across the midline (arrowheads) while AN-2 dendrites tend to respect the midline. **(B)** After one foreleg is removed (X) in an adult cricket, deafferented AN medial dendrites grow across the midline toward the contralateral N5. In addition, N5 axons on the intact side extend toward the deafferented AN dendrites. Only a small portion of total post-deafferentation N5 growth crosses the midline (arrows indicate the area where the majority of N5 compensatory growth emerges). 40× z-stack confocal images of **(C)** control, **(D)** 3 days, **(E)** 7 days, and **(F)** 14 days deafferents display general AN and N5 anatomy and increasing amounts of growth following deafferentation. In order to see clearly the dendrites at the midline, a subset of this cell's optical sections were projected. The lateral dendrites of this cell were in optical sections that were not included in this projection. The dotted lines represent approximated midlines. Scale bars = 20 μm.

as well. Given the close proximity and overlap of AN-1 and AN-2 dendrites, we will refer to these dendrites more generally as "AN" dendrites throughout. As has been established for this system (Hoy et al., 1985; Schmitz, 1989; Horch et al., 2011), the AN dendrites from a single cell remain largely on one side of the ganglion and do not cross the mid-line in significant numbers. The majority of N5 axons also roughly respect the midline, though as has been previously noted (Schmitz, 1989), several axons extend across the midline in many normal control animals (**Figures 1A,C**, arrowheads). Our double backfills confirm this anatomical arrangement in the prothoracic ganglia of adult control crickets (**Figure 1C**).

To understand how deafferentation in adulthood altered dendritic and axonal growth over time, we deafferented adult animals and backfilled both N5 and the ANs after 30 h, 3, 5, 7, 14, and 20 days. Representative backfills of a subset of these time points are shown in **Figures 1C–F**. Qualitatively, AN growth increased fairly rapidly over the midline after deafferentation, and generally appeared to reach its maximum extent within a week. The amount of N5 axonal growth beyond the midline increased somewhat after deafferentation, with a few additional axons crossing the midline. More dramatic, however, was the substantial sprouting of N5 axons from the posterior edge of the anterior portion of the N5 "claw" (see arrows in **Figures 1B–E**, and Horch et al., 2011).

QUANTIFICATION OF AN DENDRITIC GROWTH AFTER DEAFFERENTATION

To better understand the nature of this growth over time, we quantified a variety of axonal and dendritic characteristics after deafferentation. Accurately quantifying aspects of those axons and dendrites crossing the midline required that we first establish a consistent, unbiased method of identifying the midline. As noted in the methods, we developed a protocol in which we used the autofluorescence of the midline region in coded images (**Figure 2A**) to determine the midline location, which was then approximated as a straight line (**Figure 2B**). The extent to which axons and dendrites had crossed this midline was determined first by hand-tracing individual processes that had been backfilled with dye (**Figure 2C**) in control ganglia and in ganglia from each deafferentation period and then by taking a variety of measurements from these traces. The schematic in **Figure 2D** illustrates the measurements collected.

A small number of AN dendrites normally extended a short distance across the midline in control crickets ($T = 0$, **Figure 3A**); however, dendrites in deafferented crickets rapidly extended further across the midline. AN growth patterns were significantly and strikingly different in male and female crickets. In males, the maximum perpendicular extent, the longest dendrite, and the skeletal length each increased linearly following deafferentation. In females, however, growth of each of these variables increased non-linearly and were characterized by a rapid growth phase in the first 3 days followed by a plateau in length (**Figures 3A–C**; **Table 1**). In general, female data were less variable than male data (**Table 1**). While at day 0 there were no significant differences in dendritic characteristics (t-tests, each df = 10, each $p > 0.3$; see also 95% confidence intervals of linear and non-linear fits), by day 20, all dendritic characteristics were 45–110% greater in males than in females (**Table 1**).

QUANTIFICATION OF N5 AXONAL GROWTH AFTER DEAFFERENTATION

As previously noted, and as seen in the results of our present study, N5 axonal processes cross the midline in control animals far more frequently and more extensively than do AN-2 dendrites (Schmitz, 1989). However, N5 maximum perpendicular extent and longest axon did not change significantly after deafferentation over time (**Figures 4A,B**; **Table 2**), and there were no significant differences between males and females either specifically at the end-point of 20 days (**Figures 4A,B**; **Table 2**) or overall for data pooled over all time periods (Kruskal–Wallis tests, each df = 96, each $p > 0.26$). In contrast, N5 skeletal length increased linearly until day 20 for gender-pooled data (although not for male and female data analyzed separately: **Figure 4C**, **Table 2**) with no significant difference between skeletal length in males and females either at the end-point of 20 days (**Table 2**) or overall for data pooled over all time periods (Kruskal–Wallis test, df = 96, $p = 0.93$).

As we have previously described, many axons sprout from the N5 "claw" into the extra-neuropillar space, but do not cross the midline (Horch et al., 2011). As such, this axonal sprouting was not represented in our analysis above. This region of N5 growth, extending across the posterior edge of the anterior portion of the N5 claw, appears to be the location in which axons and contralateral ANs grow in close proximity and may be the locus for some of the functional recovery seen in deafferented AN neurons (**Figures 1D–F**, arrow, Horch et al., 2011). We quantified the volume of axon sprouting into this region (**Figure 5A**). Extra-neuropillar N5 sprouting increased linearly over time both in gender-pooled data and for male and female data analyzed separately (**Figure 5B; Table 2**), and there was not a significant difference between skeletal length in males and females overall for data pooled over all time periods (ANCOVA; $p_{\text{equal slopes}}$: df = 86, $p = 0.37$; $p_{\text{equal intercepts}}$: df = 87, $p = 0.35$).

AXONAL AND DENDRITIC GROWTH WERE INDEPENDENT

Some of the variation in our data could have been due to variations among animals that possess inherent environments either more or less conducive to growth. If so, there should be a correlation between the extent of growth of axons and dendrites per animal, with some animals showing poor growth for both axons and dendrites and others exhibiting extensive growth for both axons and dendrites. There was, however, no correlation between axonal and dendritic growth within each animal (**Figure 6**). Even when comparing extraneuropillar axonal growth, arguably a more sensitive measurement of total axonal growth, with AN skeletal growth, no correlation was evident (**Figure 6B**).

Given that female AN growth rates were non-linear while axonal growth rates were linear, perhaps it is not surprising that there was no correlation between axonal and dendritic growth across the whole population over time. The early burst of growth at 3 days and the subsequent plateau for female dendrites would not correlate well with the steady, linear growth rates seen in axons. Thus, we examined the correlation between axonal and dendritic growth at 5 and 7 days. At these time points, male and

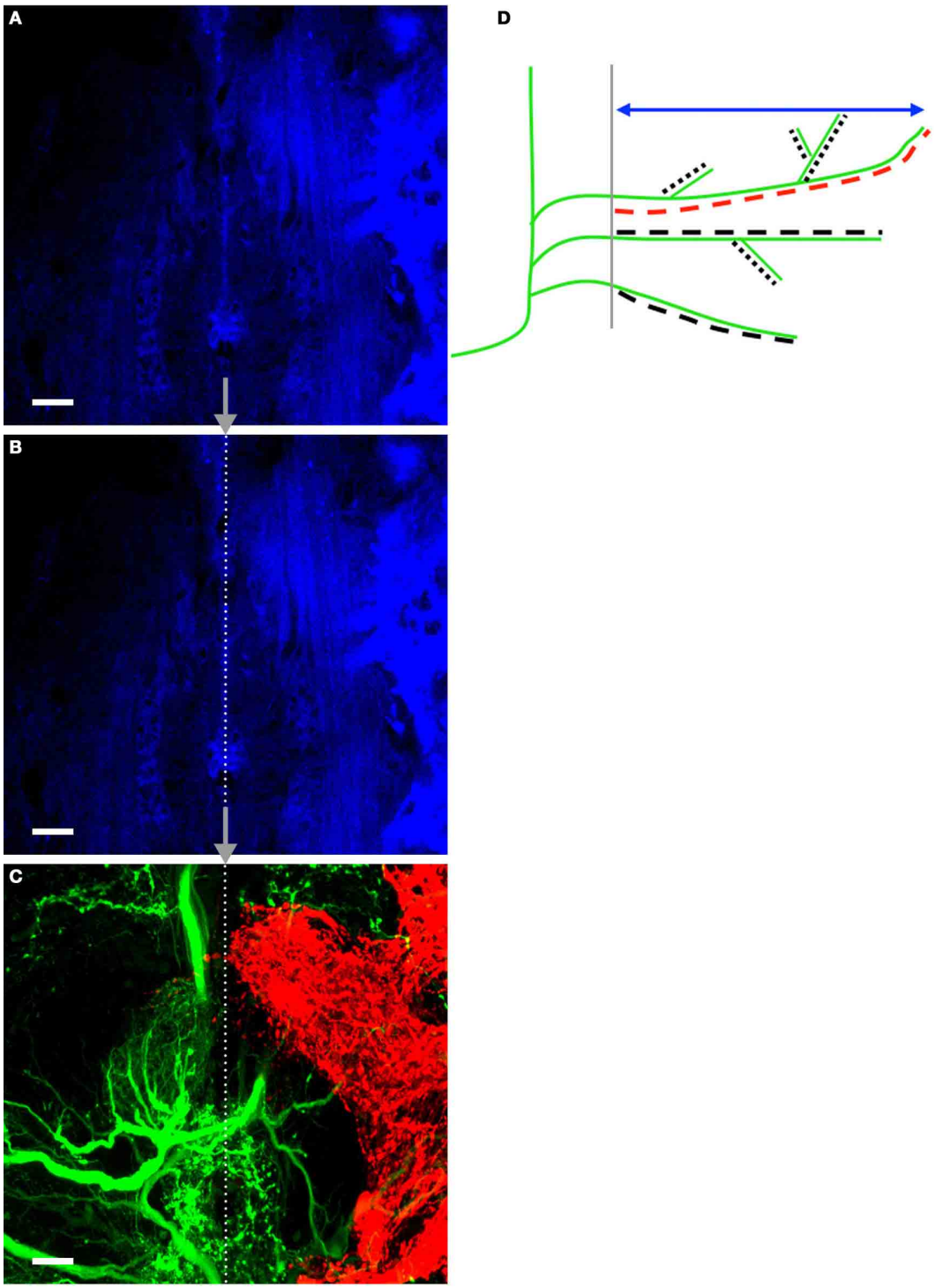

FIGURE 2 | Method for finding and placing the midline on tracing images and measurement schematic. (A) Autofluorescent cells at the midline of the prothoracic ganglia were captured in a confocal image. **(B)** Prior to hand-tracing AN and N5 processes, the midline was approximated by tracing a straight line over the autofluorescent cells. **(C)** A corresponding image of the ANs and N5 was subsequently captured. The midline was traced onto the AN/N5 image by placing the end-points of the line at the exact coordinates as they appeared on the midline image. The images shown belong to a control cricket. The midline is represented by the dotted line. Scale bars = 20 μm. A schematic highlighting the measurements made in this study is shown in **(D)**. AN dendrites crossing the midline (gray) are represented in the schematic in green. The double-headed arrow (blue) indicates the perpendicular extent measurement. The longest dendrite length was recorded for each animal and is highlighted here in red. All dendrites and their branches were hand-traced (represented by the dashed lines), and the skeletal length measurement is the sum of the lengths of the dashed lines. For simplicity AN dendrites only are represented here but the same methods were used to measure N5 axonal growth across the midline.

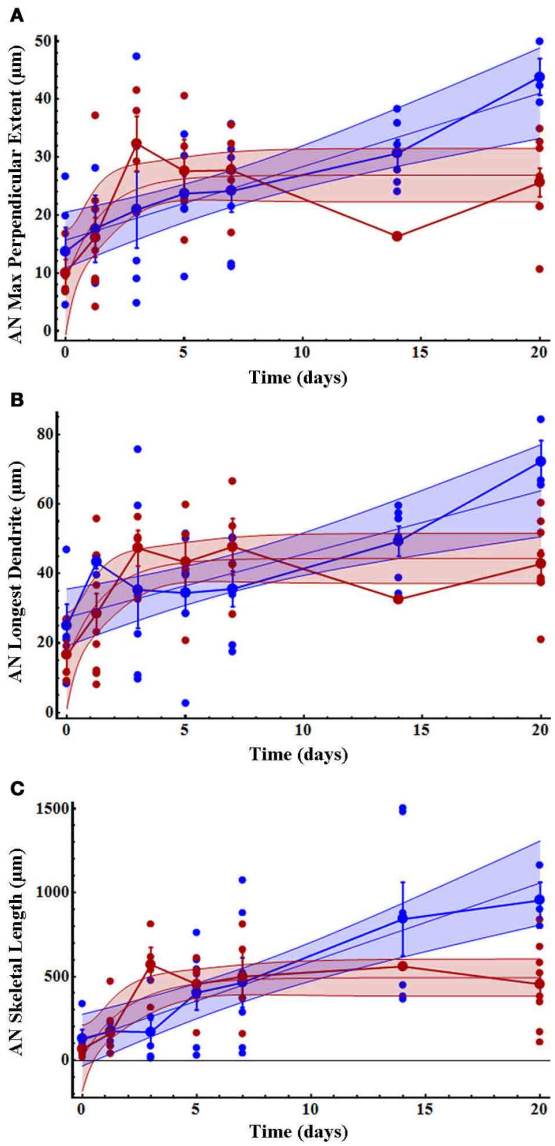

FIGURE 3 | Initial AN growth across the midline increased more rapidly in females than males over time after deafferentation. (A) AN dendritic maximum perpendicular extent, **(B)** longest dendrite, and **(C)** skeletal length increased non-linearly in females (red) and linearly in males (blue). Dendritic length in females reached a plateau by 3 days after deafferentation, while male dendritic length increased more slowly and did not plateau within the measured time period. The mean for each time point is shown as a slightly larger data point, and error bars represent standard error of the mean. The blue and red shaded areas represent 95% confidence intervals. Female AN control ($n = 4$), 30 h ($n = 9$), 3 days ($n = 4$), 5 days ($n = 4$), 7 days ($n = 7$), 14 days ($n = 1$), and 20 days ($n = 9$). Male AN control ($n = 5$), 30 h ($n = 4$), 3 days ($n = 6$), 5 days ($n = 8$), 7 days ($n = 7$), 14 days ($n = 6$), and 20 days ($n = 3$). See **Table 1** for regression equations and statistics.

female growth levels roughly overlapped (**Figure 3**). However, no correlation was evident between axonal extraneuropillar growth and AN skeletal length at these time points (**Figure 6C**). Finally, given that both dendritic and axonal growth rates were linear in males, we compared the growth rates of axons and dendrites across all time points in only the male animals. There was no significant correlation within male animals between axonal and dendritic growth over time (**Figure 6D**). These results imply that the factors that influence dendritic and axonal growth within the prothoracic ganglion are independent.

DISCUSSION

This study quantified AN dendritic and N5 axonal growth characteristics in the male and female cricket, *Gryllus bimaculatus*, to better understand general growth patterns of neurons in an adult system after deafferentation. We were surprised to find that the dendritic growth of deafferented AN dendrites was quite different between males and females. Male dendritic growth rates were linear and, on average, surpassed female dendrites in length, while female dendritic growth rates were non-linear, consisting of a burst of growth in the first 3–5 days followed by a plateau. Axonal growth, on the other hand, did not vary by sex. N5 axons did not grow further past the midline in deafferented ganglia as compared to controls but axonal skeletal length did increase linearly after deafferentation. This suggests that axons branched to form more elaborate arbors during the compensatory growth period. We suspect that branching is a major contributor to increasing skeletal length after deafferentation, in both axons and dendrites, but further analysis of branch formation is necessary to confirm this hypothesis (Pfister et al., in preparation). Extraneuropillar sprouting of axons also increased significantly and in a linear fashion in both males and females. These measurements provide a basis of comparison for future studies investigating molecular signals that underlie the growth of AN dendrites and N5 axons after deafferentation.

DIFFERENTIAL EFFECTS OF UNILATERAL DEAFFERENTATION ON DENDRITES AND AXONS

It is not entirely surprising that dendrites and axons respond differently to unilateral deafferentation. The AN dendrites are directly deafferented by the loss of their ipsilateral auditory input, whereas the contralateral axons are only indirectly affected by this loss, presumably via the local circuits within the prothoracic ganglion. In addition, axons and dendrites in control animals respond differently to the midline, with dendrites treating it more as a strict border than axons. What is perhaps more interesting is the extent of axonal sprouting observed, given the indirect influence of the deafferentation. Contralateral afferent sprouting does not occur in all deafferented systems. Following unilateral cercal ablation in the cricket and cockroach deafferented giant interneurons in the terminal ganglion and intact contralateral cercal afferents respond by strengthening the weak synapses that already exist between them. Cercal neuron morphology generally remains stable with relatively minor, inconsistent sprouting (Murphey and Levine, 1980; Volman, 1989). In situations where the deafferented interneurons do not have pre-existing contact with contralateral afferents, injury compensation requires more dramatic morphological change. Contralateral sensory sprouting across the midline following auditory deafferentation has been described in several cricket species as well as the locust, *Locusta migratoria* (Pallas and Hoy, 1986; Lakes et al., 1990; Krüger et al.,

Table 1 | Summary of variables and statistics for AN growth analysis.

Variable	Gender	Sample size	Spearman rank		Compare linear to non-linear		r^2 and fitted parameters *in* for preferred model						*t*-test comparing end point *p*(*df*)M?F
			r	1-tailed *P* (df)	ΔAICc	Probability linear model is correct	Preferred model	r^2	*k* (per day)	*m* (μm per day)	l_0(μm)	*b*(μm)	l_∞(μm)	*p* slope = 0 (df)	
AN maximum perpendicular extent	Male	37	0.61	<0.0001 (35)	2.4	0.77	Linear	0.39	na	1.3	na	15.7	na	<0.0001 (1,35)	0.003 (10) M > F
	Female	35	0.48	0.002 (33)	8.3	0.02	Non-linear	0.88	0.68	na	8.4	na	29.2	na	
AN longest dendritic extent	Male	37	0.47	0.002 (35)	2.4	0.77	Linear	0.32	na	1.8	na	27.3	na	0.0003 (1,35)	0.003 (10) M > F
	Female	35	0.48	0.002 (33)	8.2	0.02	Non-linear	0.89	0.64	na	14.9	na	44.3	na	
AN skeletal length	Male	37	0.65	<0.0001 (35)	2.0	0.73	Linear	0.46	na	46.9	na	119.4	na	<0.0001 (1,35)	0.007 (10) M > F
	Female	35	0.58	<0.0001 (33)	9.9	0.01	Non-linear	0.80	0.52	na	12.0	na	492.8	na	

Data were first analyzed using the non-parametric Spearman rank correlation to determine whether there were significant trends in each variable over time. When the Spearman rank indicated a significant correlation, the data were fit with a linear and non-linear model (Equations 1 and 2) and the Akaike Information Criterion corrected model (AICc) was used to determine the preferred model. The parameters for the preferred model are given, with units indicated in parentheses. In addition, when the linear model was preferred the probability that the best fitting line has a zero slope and the degrees of freedom of that test are given. Finally, end-points were compared via a t-test after testing assumptions of normality and equal variance.

FIGURE 4 | N5 axonal skeletal length increased significantly over time after deafferentation, while the longest axon and perpendicular extent of N5 axons did not. (A) N5 axonal maximum perpendicular extent and **(B)** longest axon showed no significant change over the deafferentation time period while **(C)** skeletal length increased significantly in a linear fashion. N5 growth patterns did not differ between males (blue) and females (red). Black data points represent means, and error bars represent standard error of the mean. The shaded gray area indicates the 95% confidence interval. Female N5 control ($n = 6$), 30 h ($n = 9$), 3 days ($n = 6$), 5 days ($n = 9$), 7 days ($n = 5$), 14 days ($n = 3$), and 20 days ($n = 10$). Male N5 control ($n = 9$), 30 h ($n = 7$), 3 days ($n = 4$), 5 days ($n = 9$), 7 days ($n = 7$), 14 days ($n = 9$), and 20 days ($n = 7$). See **Table 2** for regression equations and statistics.

2011a,b). Limited quantitative measurements of *L. migratoria* contralateral afferent sprouting indicate that the N5 sprouting that we measured in *G. bimaculatus*, although perhaps somewhat more extensive than that seen in the locust, does not appear to

Table 2 | Summary of variables and statistics for N5 growth analysis.

Variable	Gender	Spearman rank			Compare linear to non-linear			r^2 and fitted parameters *m*for preferred model				*t*-test or Kruskal–Wallis comparing end point
		Sample size	*r*	1-tailed *P* (df)	ΔAICc	Probability linear model is correct	Preferred model	r^2	*m* (μm per day)	*b* (μm)	*p* slope = 0 (df)	*p* (df) M?F
N5 max perpendicular extent	Male	52	0.101	0.24 (50)	na	na	na	na	na	na	na	0.93 (15) M = F (K–W)
	Female	46	0.094	0.27 (44)	na	na	na	na	na	na	na	
	Pooled	98	0.091	0.19 (96)	na	na	na	na	na	na	na	
N5 longest axon	Male	52	0.155	0.14 (50)	na	na	na	na	na	na	na	0.94 (15) M = F (*t*-test)
	Female	46	0.057	0.35 (44)	na	na	na	na	na	na	na	
	Pooled	98	0.102	0.16 (96)	na	na	na	na	na	na	na	
N5 skeletal length	Male	52	0.30	0.01 (50)	0.1	0.51	Linear	0.06	7.5	218.4	0.08 (1,50)	0.91 (15) M = F (*t*-test)
	Female	46	0.25	0.05 (44)	2.2	0.75	Linear	0.03	6.1	237.8	0.26 (1,44)	
	Pooled	98	0.27	0.003 (96)	2.1	0.74	Linear	0.04	6.8	227.7	0.04 (1,96)	
N5 extraneuropillar growth	Male	44	0.49	0.0003 (42)	2.3	0.76	Linear	0.24	710	6105	<0.001 (1,42)	0.52 (14) M = F (*t*-test)
	Female	46	0.36	0.008 (44)	0.13	0.52	Linear	0.11	461	9755	0.02 (1,44)	
	Pooled	90	0.42	<0.0001 (88)	1.6	0.69	Linear	0.17	579	7977	<0.0001 (1,88)	

For a description of the table columns, see the legend for ***Table 1*** *with the following addition: end-points were compared with a non-parametric Kruskal–Wallis test instead of a t-test when there were significant differences in variance around the male/female means; end-point data were always normally distributed.*

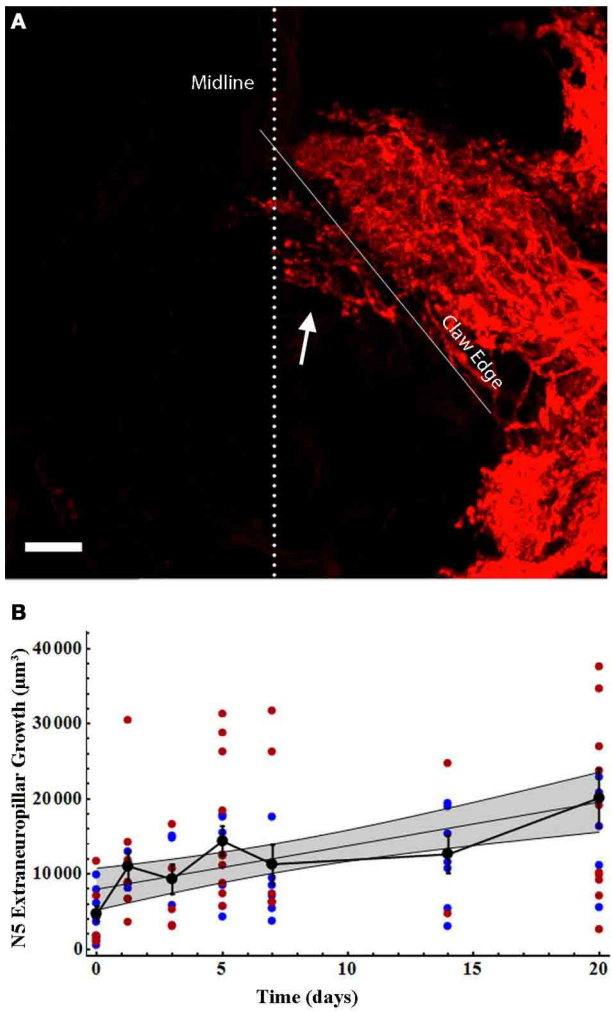

FIGURE 5 | N5 extraneuropillar growth increased in the same linear pattern for males and females following deafferentation. (A) Total N5 growth consisted of the axons crossing the midline (dotted line) as well as the axons crossing the bottom edge of the anterior portion of the claw (solid white line) to extend outside of the neuropil (arrow). The volume of this extraneuropillar growth was measured. The N5 image shown belongs to a 7 days deafferent. Scale bar = 20 µm. **(B)** Male (blue) and female (red) N5 extraneuropillar growth data sets increased linearly and were not significantly different from one another. Black data points represent means, and error bars represent standard error of the mean. Female N5 extraneuropillar growth control ($n = 7$), 30 h ($n = 6$), 3 days ($n = 5$), 5 days ($n = 10$), 7 days ($n = 6$), 14 days ($n = 2$), and 20 days ($n = 10$). Male N5 extraneuropillar growth control ($n = 9$), 30 h ($n = 6$), 3 days ($n = 3$), 5 days ($n = 7$), 7 days ($n = 6$), 14 days ($n = 7$), and 20 days ($n = 7$). The shaded gray area represents the 95% confidence interval. See **Table 2** for regression equations and statistics.

be atypical for axonal growth in the insect system (Lakes et al., 1990). After unilateral sensory ablation, input from sensory afferents on the intact side is necessary for dendritic sprouting and compensation in a number of insect systems and is required after deafferentation in the cricket (Murphey and Levine, 1980; Pallas and Hoy, 1986; Volman, 1989; Krüger et al., 2011b). However, it is not clear how deafferented dendrites and contralateral axons are stimulated to grow after deafferentation or whether axons and dendrites signal one another during the growth process. Our measurements indicated that axonal and dendritic sprouting occurred simultaneously but did not provide any hint as to whether axons are attracting dendrites, dendrites are attracting axons or if there is a third party at work.

The N5 growth that we observed crossing the midline is consistent with that previously reported (Schmitz, 1989). The distance that N5 axons extended past the midline and the length of the longest axon in controls did not change after deafferentation. However, N5 skeletal length did increase after deafferentation, indicating that N5 axons must have increased their total length by means of additional axons crossing the midline and/or branching. Although N5 growth has been observed in the adult after larval deafferentation (Horch et al., 2011), N5 extraneuropillar growth has not before been described or quantified after deafferentation in adult crickets. N5 extraneuropillar growth is of particular interest because these axons emerging from the middle region of the claw appear to be in close proximity to AN dendrites, while the axons extending from the anterior-medial portion of the claw across the midline are not in a position to physically interact with sprouting AN dendrites (cf. **Figure 1E**). Because of the dense and intricate N5 axonal morphology, and because all N5 axons have been backfilled, it is impossible to determine from where exactly the extraneuropillar sprouting originated within N5. However, the compensatory axonal growth extended out, or through, the region of N5 known to contain both low (5 kHz) and ultrasound specific afferents which would appropriately excite AN-1 an AN-2, respectively, (Imaizumi and Pollack, 2005).

The amount of extraneuropillar growth varies extensively among animals (**Figure 5B**). Given that the formation of synapses is thought to stabilize axon branches (Ruthazer et al., 2006), perhaps stochastic differences in the rate of contact between axons and dendrites, and thus in synapse formation, results in highly variable growth profiles for axons in different animals. Additional auditory interneurons integral to this circuit, such as the Omega neuron, also show compensatory dendritic growth upon unilateral deafferentation (Schildberger et al., 1986). The formation of synapses with post-synaptic partners other than the ANs likely contributes to the overall variation in N5 growth as well, although this has not been studied. Regardless, it is logical to think that the amount of extraneuropillar growth might correlate with the variations in the functional recovery of one-eared animals (Schmitz, 1989), but such a hypothesis has never been tested.

Dendrites in the cricket auditory system, on the other hand, do grow extensively and rapidly across the midline both in males and females. Extensive dendritic growth appears to depend on the availability of contralateral afferents. In the cercal system interneurons already share some contact with contralateral afferents, and thus dendritic morphologic change is generally very subtle after deafferentation, although scattered dendritic sprouting across the midline has been reported for the cricket *Acheta domesticus* (Murphey et al., 1975). In the locust flight system, interneurons share synaptic connections with contralateral afferents while flight motor neurons do not. Following single hindwing tegula ablation, unilaterally deafferented motor neurons will send dendrites across the midline, likely to gain input from

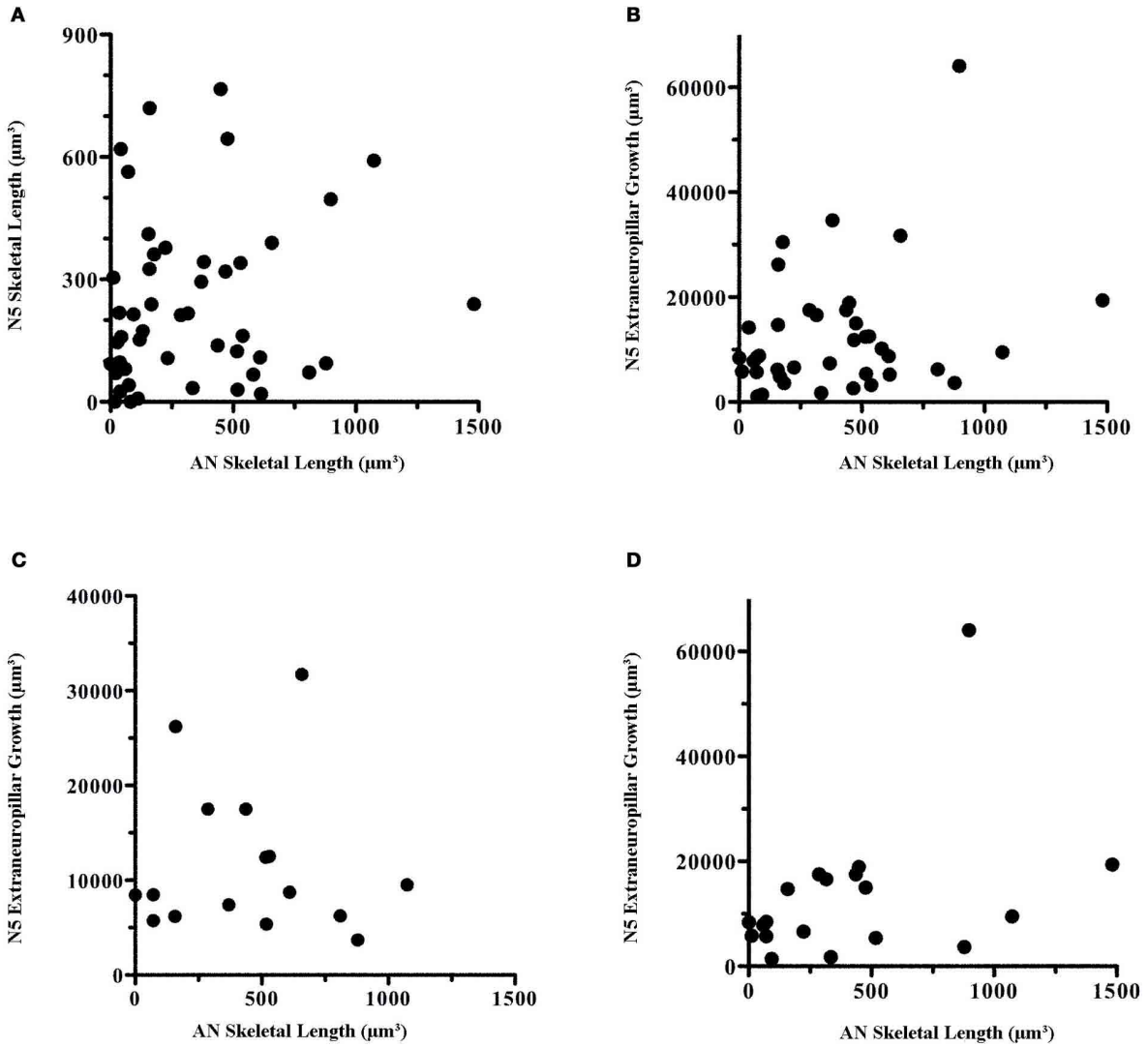

FIGURE 6 | There was no correlation in the amount of dendritic growth in comparison to axonal growth within individuals. Within-animal AN skeletal length was compared with **(A)** N5 skeletal length, **(B)** N5 extraneuropillar growth, and **(C)** N5 extraneuropillar growth at 5 and 7 days. **(D)** Male N5 extraneuropillar growth at all time points also did not correlate with male AN skeletal length. These data were analyzed by Spearman rank correlation but there were no significant correlations among any of the comparisons (**A**: $p = 0.16$, $n = 48$; **B**: $p = 0.21$, $n = 39$; **C**: $p = 0.88$, $n = 16$; **D**: $p = 0.10$, $n = 19$).

contralateral afferents, while neither the contralateral afferents nor interneurons in this case undergo major sprouting (Wolf and Büschges, 1997). After bilateral hindwing ablation in the locust, interneurons have lost both ipsilateral and contralateral input resulting in more extensive interneuron dendritic growth as these neurons seek out forewing afferent input (Büschges et al., 1992). AN interneurons in the cricket do not have contralateral afferent connections prior to deafferentation and thus require extensive growth and rearrangement to receive contralateral input as has been demonstrated in different cricket species (Hoy et al., 1985; Pallas and Hoy, 1986; Brodfuehrer and Hoy, 1988; Schmitz, 1989).

Given that previous studies had noted very few dendrites crossing the midline in controls (Hoy et al., 1985; Schildberger et al., 1986; Brodfuehrer and Hoy, 1988; Schmitz, 1989), we were somewhat surprised to find measurable dendritic midline crossing in control ganglia. However, our approximation of the midline as a single line is probably simplistic. In fact, the glia of the midline do have some width, and it is possible that some of the dendritic growth evident in controls would be reduced if the midline width were better understood. Previous research (Schmitz, 1989) qualitatively described that AN growth in females continuously progressed over time but far more slowly than our measurements indicated. AN-2 was previously reported not to have crossed the midline by 3 days after deafferentation and to have only reached maximum growth past the midline at 27 days after deafferentation. Our observations reported here clearly indicate that AN dendrites not only generally crossed the midline by 3 days, but may even have approached maximum growth by 3–5 days after deafferentation particularly in

females (**Figure 3**). Presumably consistent midline identification, the use of fluorescent tracers and three-dimensional confocal images enhanced the ability to detect small changes in growth.

SEXUALLY DIMORPHIC DENDRITIC GROWTH IN RESPONSE TO INJURY

We were surprised to find such striking differences in AN dendritic growth rates between male and female adult crickets. If our backfills had included only AN-1, which responds best to the calls of conspecifics, this sexual dimorphism might be expected since males are worse at discriminating between calling songs than females are (Pollack, 1982). However, we believe our images consist predominantly of AN-2 dendrites, which are primarily involved in mediating negative phonotaxis to bat ultrasound calls, with very little contribution of AN-1 dendrites. Assuming that we are looking mainly at AN-2 dendrites, one would not necessarily expect to find varying compensatory growth rates between males and females. AN dendrites in females reached their maximum perpendicular extent, formed their longest dendrite, and completed total arbor growth often by 3 days after deafferentation. Growth then stabilized by 5 days after deafferentation in females. Male AN dendrites, on the other hand, grew more slowly and steadily than female dendrites, but eventually they grew on average twice as long, twice as far beyond the midline, and consisted of twice as much skeletal length as AN-2 female dendritic arbors.

Physiological studies in *Teleogryllus oceanicus* show that functional recovery of ultrasound responses occurs between 4 and 6 days after deafferentation, though tuning curves do not reach control levels even by 28 days after deafferentation (Brodfuehrer and Hoy, 1988). The physiological response strength of AN-2 neurons in females increases over time and in correlation with the increasing number of dendrites crossing the midline (Schmitz, 1989). Physiological and behavioral recovery have not yet been compared between males and females. It is unclear whether females require less AN-2 growth, thus forming more efficient connections with the contralateral N5, than males to regain the same strength in physiological response or if greater total dendritic growth allows males to regain stronger AN-2 physiological responses.

What might cause these sexually dimorphic growth rates in response to injury? Hormones are obvious candidates for creating dimorphic dendritic growth responses. Hormones can influence dendritic and axonal structure both in vertebrates and invertebrates (DeVoogd and Nottebohm, 1981; Cooke and Woolley, 2005; Williams and Truman, 2005). In insects, ecdysteroids, which drive metamorphosis and act as sex hormones, are thought to influence dendritic and axonal growth and branching (Bowen et al., 1984; Truman and Reiss, 1988, 1995; Weeks and Ernst-Utzschneider, 1989; Levine et al., 1991; Prugh et al., 1992; Kraft et al., 1998; Matheson and Levine, 1999; Cayre et al., 2000; Williams and Truman, 2005; Horch et al., 2011). For example, during metamorphosis in *Manduca* and *Drosophila*, certain classes of CNS motor neurons dramatically reorganize their dendritic arborizations in response to shifts in hormones (Duch and Levine, 2000; Consoulas et al., 2002). Dendritic growth during metamorphosis appears to be initiated by low Ca^{2+} levels, which stimulate growth cone formation (Duch and Levine, 2000), and these low Ca^{2+} levels are, at least in part, under ecdysteroid regulation (Grünewald and Levine, 1998).

It is possible that the differences in AN male and female growth in the cricket could be attributed to different hormonal environments. Far greater ecdysone titers have been described in adult *Gryllus firmus* female crickets as compared to males, though the exact levels depend on life history choices (Zera et al., 2007). While ecdysone titers have not been compared directly in adult male and female *G. bimaculatus*, it is reasonable to think that similar differences might exist between males and females in this species (A. Zera personal communication). Furthermore, it is possible that the same hormonal stimulus could influence male and female neuronal morphology in very different ways, as has been demonstrated in mammals (Cambiasso et al., 1995, 2000; Carrer et al., 2005). This indicates that male and female neurons can have inherent differences that may be due, at least in part, to sex chromosome-related gene expression as well as the developmental hormonal environment (Hutchison, 1997; Agate et al., 2003; Dewing et al., 2003). Unlike systems of dramatic sexual dimorphism, we presume that AN-2 reorganization is meant to accomplish the same goal in both males and females: that of regaining the ability to localize bat ultrasound. However, it is not known whether females and males recover AN-2 physiological function at the same time or if the amount of AN-2 growth corresponds to the extent of physiological recovery in either males or females.

AN-2 is tuned to respond to pitches higher than 15 kHz and thus is often thought of as the "bat ultrasound detector." If indeed the rapid dendritic growth in females demonstrated here is due predominantly to AN-2 dendritic growth, and presuming that this growth corresponds to more rapid functional recovery, why might females need to recover ultrasound detection more rapidly than males? It is possible that females have a higher risk of encountering predatory bats as females spend more time flying at night while searching for males, who are singing in front of their burrows in the ground. Alternatively, it is possible that AN-1 dendrites principally contribute to this sexually dimorphic response. Though both males and females need to respond to conspecific calls, it seems reasonable that females might need to recover this ability more rapidly than males. Though we feel confident that the majority of the dendrites measured in our images are AN-2 dendrites, it would require physiological methods to cleanly document the sexually dimorphic recovery of function in both AN-1 and AN-2 separately.

IMPLICATIONS FOR THE SEARCH FOR COMPENSATORY GROWTH TRIGGERS

One of the reasons to carefully quantify dendritic and axonal growth after deafferentation is to try to gather hints about what types of factors might be triggering the unusual compensatory growth in the cricket. Given that N5 axons and AN dendrites grow so differently, it is unlikely that there is a single trigger that induces these changes. In fact, the complete lack of correlation between dendritic and axonal growth strongly implies that separate sets of factors influence axonal and dendritic growth independently. It is likely that a complex milieu of factors is created upon deafferentation, which separately instruct or guide axonal and

dendritic growth, and we have some evidence that multiple factors are indeed regulated upon deafferentation (Horch et al., 2009). Furthermore, the results presented here indicate that these factors might interact with hormones or other sex-dependent factors to influence dendritic growth. At a simplistic level, axonal and dendritic growth after deafferentation can be classified into four stages (1) midline crossing; (2) process extension; (3) process elaboration or branching; and (4) synapse formation. These stages are not necessarily discrete nor are they likely to occur in strict chronological order.

Midline regulation is a fundamental component of neuronal organization during development and involves well-conserved molecular mechanisms from worms and flies to mammals. Recent experiments have found that many of the factors that were first described as axon guidance factors also influence the guidance of dendrites (Furrer et al., 2003, 2007; Mauss et al., 2009; Hocking et al., 2010; Teichmann and Shen, 2011). Preliminary evidence indicates that the midline regulator protein, Slit, and the Slit receptor, Robo, do exist in the cricket *G. bimaculatus* (unpublished observations), though no evidence has yet confirmed whether Slit is expressed at the midline. The family of Slit proteins can persist in the adult CNS, as has been demonstrated in rats but the levels and areas of expression during development generally change once the animal reaches adulthood (Marillat et al., 2002). It is possible but not certain that AN dendrites are overcoming a midline barrier as they cross the midline after deafferentation in the adult. The process by which neurons in an adult system are allowed to cross a molecular barrier deserves further study, and understanding this process could contribute to our general understanding of the inhibition of neuronal growth after injury, which is frequently the outcome in most other animal systems.

Though process extension, branching, and subsequent synapse formation are likely influenced by a rich variety of factors, the semaphorin family of neuronal guidance molecules, in theory, could influence all these stages. Semaphorins were first described as chemorepellents for axons (Luo et al., 1993), though they have subsequently been shown to influence the guidance, outgrowth, branching, and targeting of dendrites as well (Polleux et al., 2000; Fenstermaker et al., 2004; Shelly et al., 2011; Sweeney et al., 2011). Semaphorins have also been shown to influence synapse formation during development (Godenschwege et al., 2002; Ding et al., 2011). While the post-developmental roles for semaphorin are less clear, there is good evidence that semaphorin expression levels are regulated after injury in adult mammals (De Winter et al., 2002; Ara et al., 2004; Hashimoto et al., 2004; Kopp et al., 2010). Semaphorin 2a (*Sema 2a*), a secreted semaphorin, has been identified in *G. bimaculatus* (Maynard et al., 2007) and is expressed in the prothoracic ganglion (unpublished observations). Investigation of the differential expression of semaphorins, as well as additional ligands and receptors, will shed light on the mechanisms responsible for this compensatory phenomenon within the adult cricket auditory system.

Research on compensatory neuronal growth in adult crickets is still limited, but the field cricket provides a promising system to better understand fundamental dendritic and axonal growth characteristics that might offer insight into the general mechanisms that trigger neuronal growth in adults. In addition, more strict attention to the possible differences between male and female nervous system function, injury response and recovery at the cellular level, and the interaction of these processes with hormones will likely reveal new insights into how neuronal systems recover from injury.

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Lesion-induced insights in the plasticity of the insect auditory system

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The auditory networks of Orthoptera offer a model system uniquely suited to the study of neuronal connectivity and lesion-dependent neural plasticity. Monaural animals, following the permanent removal of one ear in nymphs or adults, adjust their auditory pathways by collateral sprouting of afferents and deafferented interneurons which connect to neurons on the contralateral side. Transient lesion of the auditory nerve allows us to study regeneration as well as plasticity processes. After crushing the peripheral auditory nerve, the lesioned afferents regrow and re-establish new synaptic connections which are relevant for auditory behavior. During this process collateral sprouting occurs in the central nervous networks, too. Interestingly, after regeneration a changed neuronal network will be maintained. These paradigms are now been used to analyze molecular mechanism in neuronal plasticity on the level of single neurons and small networks.

Keywords: auditory systems, plasticity, regeneration, axotomy

Like other neural systems in insects, auditory networks have long been regarded as inflexible neural systems. A main basis for this impression is that in some taxa, like Orthoptera, auditory systems are involved in intraspecific communication whose signals are relatively constant in a population. These acoustic signals facilitate species discrimination which implies reliable neuronal processing of such signals. Orthopteran auditory systems are divers, but they have in common that about 20–80 primary sensory neurons are located in the peripheral ear. The sensory axons project ipsilaterally into a target neuropile in the respective ganglia of the central nervous system (CNS; **Figure 1**; Stumpner and von Helversen, 2001). Within the neuropile the afferents are monosynaptically connected to individually identifiable first order interneurons. These interneurons process and transmit the information either to the contralateral side or to higher centers in the CNS. The different auditory systems are quite well-known in their anatomy, physiology, and behavior in intact animals, allowing to analyze lesion-induced neuronal plasticity. Two different experiment approaches have been used. Firstly, the information transfer has been interrupted permanently with a removal of one ear. Anatomical, physiological and behavioral analyses show astonishing neuronal plasticity in the auditory system. Secondly, a transient lesion, an axotomy of the auditory afferents has been used to study a combination of regenerative and plasticity processes. Here the results of lesions in auditory systems of Orthoptera are reviewed in respect to the two paradigms and in respect to the first data on molecular mechanisms behind the processes.

PERMANENT LESION

Adult insects cannot regenerate lost organs. Therefore a loss of an ear with its sensory cells results in a permanent interruption of auditory afferent activity on one side of the CNS. Consequently, an asymmetry of information processing takes place which, for example, influences the directionality of the auditory system in the CNS. In many Orthoptera, phonotactic behavior can be used for analysis of changes and plasticity in auditory system. During phonotaxis monaural individuals typically move toward the intact side (side of highest excitation), resulting in circling instead of a linear approach to a sound source. Nevertheless, these insects might reach the sound source, as has been documented for crickets and bush crickets (Huber, 1987; Lakes and Mücke, 1989; Schmitz, 1989). It could even be shown that the directionality might improve with time, despite an ongoing changed excitation balance in the nervous system (Schmitz, 1989). Such changes are clearly indicative for plasticity processes in the afferent pathways of adult insects. During this process, interneurons connected monosynaptically to afferents might sprout collaterals and establish new synaptic connections (Schildberger et al., 1986; Schildberger and Kleindienst, 1989; Schmitz, 1989; Lakes et al., 1990). Interestingly, these interneurons typically sprout into the contralateral intact neuropil, although how the process is initiated and how the target is recognized remains poorly understood. Recent work has identified first molecular factors during this process and it could be shown that the guidance molecule *sema2a* is upregulated during dendritic growth (Horch et al., 2009, 2011). Collateral sprouts can also cross the midline, whereas in embryonic development regulatory sequences direct fiber growth across the midline. It will be interesting to see if and how factors like *slit* or *robo* are involved in controlling adult neuronal morphology and collateral sprouting after lesion. So far, neither in the adult locust nor in the Drosophila CNS an expression of *slit* or *robo* could be immuncytochemically detected up to 48 h after lesion (Lakes-Harlan et al., unpublished). Perhaps the factors

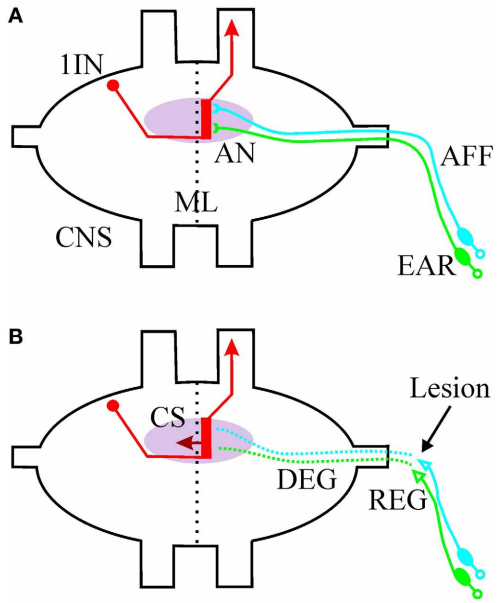

FIGURE 1 | Schematic drawing of the afferent auditory system of Orthoptera (exemplified for Ensifera). (A) The primary sensory neurons are located in the peripheral ear and project their axons (AFF, afferents) to an auditory neuropile (AN, pink) in the central nervous system (CNS). The afferents synapse onto the dendritic region (red bar) of first order auditory interneurons (1IN). The processing is strictly ipsilateral of the midline (ML). The interneurons could transport the information to the contralateral side (not shown) and/or toward higher centers in the CNS. **(B)** After a peripheral lesion of the afferents (arrow), the distal part of the axon degenerates (DEG) and regeneration starts at the proximal part (REG). First order interneurons grow collateral sprouts (CS) across the midline into the intact part of the auditory neuropile.

are important only during embryonic development, raising the question how collateral sprouting is regulated in adults.

Typically only interneurons monosynaptically connected to the lesioned afferents react with a sprouting. Why do only deafferented interneurons react (and to a small part contralateral auditory afferents)? Molecular cues from degenerating afferents will be present for all neurons in the neuropile and adjacent areas. Neurotrophic factors comparable to vertebrates are not known in insects, so it will be interesting to analyse anterograde or retrograde signals at insect synapses. Due to the permanent removal of one ear, the corresponding neuropile shrinks in volume (Krüger and Lakes-Harlan, 2010). The neurochemical composition of the neuropile area, however, is largely unchanged. The histochemical staining intensity for acetylcholinesterase is decreased by less than 10%, whereas with immunocytochemistry no change was found for γ-amino butyric acid and serotonin (Krüger and Lakes-Harlan, 2010). The synapse specific vesicle-associated membrane protein (*vamp*) is downregulated 7 days after deafferentation (Horch et al., 2011).

A lost ear is not completely regenerated even if the lesion happened in the first larval instar. Crickets have a rather good regeneration capacity of peripheral structures; however, even in these insects a complete and functional regeneration of the complex hearing organ has never been demonstrated (Huber, 1987). Crickets which have regenerated a seemingly entire leg will still only regenerate parts of the auditory system, like tympanal membranes and a few sensory units. Hearing is always impaired due to the incomplete rebuilding (Huber, 1987). Beside the complexity of the sensory organ, the failure of functional regeneration might also be due to the type of sensory cell. Auditory receptors of insects belong to scolopidial sensory units. Each unit consists typically of a sensory cell, a glial cell, an attachment cell, and a scolopidial cell. The scolopidial cell forms the characteristic scolopale, which surrounds the sensory dendrite. Although these scolopidia share many developmental pathways with other peripheral sensory sensilla, they regenerate differently. Hair sensilla embedded in other regenerating epidermal tissue regenerate rather easily (Lüdke and Lakes-Harlan, 2008). By contrast scolopidial units forming complex sensory organs are only rarely regenerated in Orthoptera. These differences in regeneration of sensory neurons are not understood so far, but might be related to general regenerative capabilities. In phasmids, which have a huge regeneration capability of appendages, scolopidial organs are regenerated inside the legs (Friedrich, 1930).

TRANSIENT LESION

A transient lesion in the auditory afferents starts regenerative processes in addition to possible plastic processes in the central auditory network. A typical transient lesion is an axotomy, performed either as tympanal nerve crush or as nerve cut. No mayor differences in respect to regeneration capacity between crushed and cut axons have been reported in insects. Many studies use nerve crush as an experimental paradigm, because a crushing usually leaves the nerve sheath intact and the sheath might function as a guiding structure. Such operations have been performed on the tympanal nerve in different species of Orthoptera (Pallas and Hoy, 1988; Jacobs and Lakes-Harlan, 2000) and in all cases a neuronal regeneration of the sensory axons occurred. The axotomy did not elicit an immediate change in the electrical response properties of the axons, although some minutes after the operation the number of action potentials per acoustic stimulus might increase (Lakes-Harlan, 2004). The axotomy divides the neuronal fiber in two parts: the proximal part remains intact and a Wallerian degeneration back to the cell bodies has not been observed in insects. The distal part of the auditory axon (i.e., the central arborization) that is separated from the cell body degenerates (**Figure 1B**). Such degeneration in not self-evident as isolated axons in insects may survive disconnected from the cell body for a long time. In very small insect species anucleate processes might even be regular parts of neuronal networks (Polilov, 2012). In the CNS of locusts a fragmentation, but no degeneration, of lesioned axons by glial cells has been observed (Jacobs and Lakes-Harlan, unpublished). By contrast, in the peripheral auditory system the degeneration has been demonstrated ultrastructurally (Jacobs and Lakes-Harlan, 1999). Glial cells are involved in the degeneration process, and markers for glial cells, like glial cell binding lectins, show a temporary increased binding in the auditory neuropil (Jacobs and Lakes-Harlan, 1997). The degeneration takes about 3–5 days, but the afferent pathway to the CNS and within the CNS might contain degeneration products

and an increased number of glial cells processes for a much longer time. Therefore, the axonal pathway is likely to contain a distinct molecular signature that might be used for guided regeneration.

The proximal part of the axon and the cell soma can start regenerative growth (**Figure 1B**). Probably not all, but a significant number of sensory neurons regenerate neuronal processes after a crush in both, adults and juveniles, respectively. Shifts in the hearing threshold curves during regeneration indicate that not all fibers regenerate (Krüger et al., 2011a). The initial signal for regrow is not yet determined in insects. Immunohistochemically, an upregulation of the transcription factor p53 expression, but not of CREB (cAMP response element-binding protein) could be shown in sensory neurons after tympanalnerve lesion in *Schistocerca gregaria* (Sohn and Lakes-Harlan, unpublished). In the brain of *Drosophila* factors like c-Jun N-terminal protein kinase are involved in axonal regeneration and target recognition (Ayaz et al., 2008).

At the tip of the regenerating nerve, actin and tubulin seems to be upregulated (evidenced by immunhistochemistry on lesioned tympanal nerves of adult *Schistocerca gregaria*; Sohn and Lakes-Harlan, unpublished). The next step during regeneration involves neuronal pathfinding. Regenerating axons often follow the original paths; however, they might leave these pathways or use other pathways from the beginning. This indicates that degeneration products are not necessary for pathfinding, although they might support it. In sensory systems a key guidance molecule could be the homophilic cell adhesion molecule Fasciclin I. This cell surface molecule is expressed on probably all sensory axons of insects during embryogenesis (Bastiani et al., 1987); its expression is maintained in the adult nervous system in distinct sensory neuropil areas, but not in others (Jacobs and Lakes-Harlan, 2000). Upregulation of this molecule has been demonstrated in neuronal regeneration in the antennal system, but not yet for auditory axons (Stern et al., 2012). However, it has been documented that regenerating auditory afferents can follow Fasciclin I positive non-auditory axonal tracts (Jacobs and Lakes-Harlan, 2000). But eventually, such misled auditory afferents leave the false tracts and reach the correct target area. The regenerated fibers arborize only within the target auditory neuropile. The molecular cues for recognizing the target area and for the development of specific terminal arborizations are not known. Typically a regenerated axonal projection shows some anatomical plasticity as it has distinct features, like longer collaterals, discriminating it from a normal projection. In many cases the projection area is enlarged, as proven for regenerated fibers from hair sensilla on the locust leg (Lüdke and Lakes-Harlan, 2008). Interestingly, the regenerated projection is independent from lesions on contralateral afferents (Krüger and Lakes-Harlan, 2011). Such double lesion experiments were designed in order to detect factors influencing the projection pattern. First an axotomy was performed on one side and later the contralateral ear was removed. However, this secondary deafferentation of the auditory neuropile did not provide cues strong enough to trigger the ipsilateral regenerated fibers to cross the midline or to show a different regeneration pattern (Krüger and Lakes-Harlan, 2011), despite the fact that deafferentation can modulate afferent sprouts in crickets (Horch et al., 2011).

A question often raised in regeneration process is, whether developmental processes are recapitulated. In embryos of locusts the pathfinding of auditory afferents has been described, and in contrast to regeneration, no irregular fiber growth (like a trial-and-error growth) on the way to the target area could be found (Schäffer and Lakes-Harlan, 2001). During embryonic development the afferents as well as the target area express Fasciclin I which might be important for axonal guidance. Thus, the processes during development and regeneration are different.

The regenerated fibers synapse onto auditory interneurons in the target neuropil. Such regenerated synapses are functionally relevant for auditory information processing; however, not all behavioral functions are restored. In the grasshopper *Chorthippus biguttulus* regenerated fibers contribute to the discrimination of the direction of auditory signals, but not to the recognition of species specific sounds (Lakes-Harlan and Pfahlert, 1995). For a directed phonotactic reaction the animals have to recognize the species specific sound signal *and* the direction. For the sound recognition an intact ear was always necessary; after lesions on both ears, recognition of species specific sounds failed. The same lack of complete functional recovery has recently been observed in the tettigoniid *Mecopoda elongata* (Friedrich and Lakes-Harlan, unpublished). Either the regenerated synaptic connections are not specific enough, or a plasticity reaction to the transient deafferentation has changed the neuronal networks permanently. Perhaps even a combination of both possibilities takes place during regeneration. It is likely that interneurons of the network form aberrant connections in response to the lesion (**Figure 1B**). These modified connections are based on collateral sprouting and have been shown in various species (Pallas and Hoy, 1988; Lakes and Kalmring, 1991; Krüger et al., 2011a). Additionally, evidence has been found that these connections do not re-change to normal after reinnervation. Compensatory sprouting is independent from the stage of the operation and for physiological compensation of deafferentation no age related differences between nymphs and adults could be found (Krüger et al., 2011b).

Sprouting of auditory interneurons and regeneration of afferent shows that growth is possible in the CNS. However, as in vertebrates regenerative growth capabilities in insects might be different in the peripheral nervous system and in the CNS. The environment in the peripheral nerve allows regeneration of sensory axons in all investigated cases. In the CNS, interneuronal axonal regeneration could be shown in the locust embryo (Pätschke et al., 2004; Stern and Bicker, 2008), but in other cases little regeneration capacities have been found (Ayaz et al., 2008). However, insects have started to provide model systems for neuronal regeneration and plasticity, as factors like erythropoietin, nitrogen monoxide, VAMP and c-jun N-terminal protein kinase could be found to influence or regulate these processes (Ayaz et al., 2008; Stern and Bicker, 2008; Ostrowski et al., 2011).

OUTLOOK

Recent results show the immense capacities of neuronal growth, target recognition, synapse formation, and compensatory plasticity after lesion in the auditory system of insects. The auditory system could offer new insights into plasticity, as the neuronal networks are rather well-known and lesions can be

easily performed. Physiological and behavioral plasticity can be investigated with different experimental paradigms. With the further advent of molecular tools, regulatory mechanisms in the CNS will be unraveled in the locust or cricket. Approaches like differential display PCR which already indicated that regenerating tympanal sensory neurons in locusts express other genes than control neurons (Jacobs, 1997) are needed in the future. Insects provide an important platform for such studies, especially as neuronal regeneration is generally not suppressed by inhibitory factors, like myelin in mammals. Nevertheless, insect neurons normally grow during development and seem to remain stable in the adult. A lesion, however, might trigger collateral growth. What are the decisive factors which influence the balance between dynamic growth and stable morphology? The identified neurons of the auditory system are likely to offer a suitable tool to answer this question in the near future.

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Trace conditioning in insects—keep the trace!

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Trace conditioning is a form of associative learning that can be induced by presenting a conditioned stimulus (CS) and an unconditioned stimulus (US) following each other, but separated by a temporal gap. This gap distinguishes trace conditioning from classical delay conditioning, where the CS and US overlap. To bridge the temporal gap between both stimuli and to form an association between CS and US in trace conditioning, the brain must keep a neural representation of the CS after its termination—a stimulus trace. Behavioral and physiological studies on trace and delay conditioning revealed similarities between the two forms of learning, like similar memory decay and similar odor identity perception in invertebrates. On the other hand differences were reported also, like the requirement of distinct brain structures in vertebrates or disparities in molecular mechanisms in both vertebrates and invertebrates. For example, in commonly used vertebrate conditioning paradigms the hippocampus is necessary for trace but not for delay conditioning, and *Drosophila* delay conditioning requires the Rutabaga adenylyl cyclase (Rut-AC), which is dispensable in trace conditioning. It is still unknown how the brain encodes CS traces and how they are associated with a US in trace conditioning. Insects serve as powerful models to address the mechanisms underlying trace conditioning, due to their simple brain anatomy, behavioral accessibility and established methods of genetic interference. In this review we summarize the recent progress in insect trace conditioning on the behavioral and physiological level and emphasize similarities and differences compared to delay conditioning. Moreover, we examine proposed molecular and computational models and reassess different experimental approaches used for trace conditioning.

Keywords: trace conditioning, insects, stimulus trace, learning, olfaction

INTRODUCTION

Actions may have delayed, rather than immediate consequences. If you have ever woken up with a terrible headache the morning after drinking too much tequila, you probably had a feeling of nausea the next time you encountered the taste and smell of tequila and may have refrained from drinking it. Although there was a temporal dissociation between the two events—the stimulus and the negative consequence—an aversive association was formed.

To account for the possibility to associate stimuli which are separated in time, the preceding conditioned stimulus (CS) must induce a representation in the form of a stimulus trace in the neuronal network, which persists for a time after the stimulus has terminated. The stimulus trace can then be associated with the following reinforcing unconditioned stimulus (US). This form of learning where CS and US are separated by a temporal gap is termed trace conditioning, in contrast to delay conditioning, where both CS and US occur with a temporal overlap (**Figure 1A**). We refer to the CS–US interval as the time span between CS onset and US onset, while the time span between the CS offset and US onset is termed gap (**Figure 1A**).

The capability to associate two temporally separated stimuli is vital for animals, since in nature the cause and effect of a behavior are often not contiguous. Additionally, the perceived features of objects are constantly changing with time and location. Animals must memorize and integrate these changing features for object identification and tracking. For example, when a bee approaches a flower, the flower's shape and color may change drastically in the bee's perception. Still, the bee should learn to associate the initial visual stimulus with the food reward in order to initiate the approach next time (Opfinger, 1931; Menzel, 1968; Grossmann, 1970).

In 1927, Ivan Pavlov had already noted that dogs responded with increased saliva production to the CS alone after training with a whistle-sound CS and a food reward US, which were separated by several minutes (Pavlov, 1927). In this case, the salivary response was delayed proportional to the interval between the CS and the US, suggesting that the dogs learned to anticipate the US.

Since then, trace conditioning was shown in a variety of paradigms including eyeblink conditioning (Smith et al., 1969), fear conditioning (King, 1965), autoshaping (Gibbon et al., 1977), and conditioned taste aversion (Barker and Smith, 1974) using different CS (auditory, visual, gustatory) and US (food, water, shock, LiCl) in organisms including dogs, rats, pigeons, rabbits, and humans (reviewed in Rescorla, 1988).

Are the memories formed during delay and trace conditioning governed by different neuronal pathways? In mammals, for example, the hippocampus is additionally required for trace but not for delay eyeblink conditioning (Solomon et al., 1986; Woodruff-Pak and Disterhoft, 2008) and the state of awareness can play a

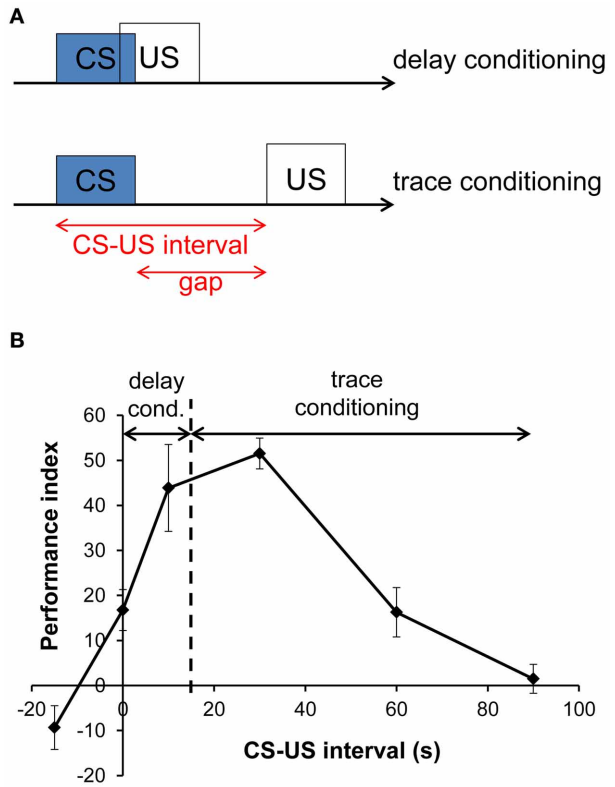

FIGURE 1 | Experimental design and memory performance in delay and trace conditioning. (A) Experimental design of delay and trace conditioning. In delay conditioning, the conditioned stimulus (CS) and the unconditioned stimulus (US) overlap, whereas they are separated by a gap in trace conditioning. The time between the onset of the CS and the onset of the US is termed CS–US interval. **(B)** Memory performance after aversive olfactory conditioning in *Drosophila melanogaster* as a function of CS–US interval. Positive and negative scores indicate conditioned avoidance and conditioned approach, respectively. A 15 s long odor presentation serves as CS. Its end is indicated by a dashed line. The US consists of four electric shocks at 90 V applied within 16 s. Trials with a CS–US interval ≤15 s are termed delay conditioning and those with a CS–US interval >15 s are termed trace conditioning. Adapted from Tanimoto et al. (2004) with changes and with permission from the author.

role for trace conditioning in humans (Clark and Squire, 1998; Clark et al., 2002; Christian and Thompson, 2003). Therefore, trace memory is proposed to be qualitatively distinct from delay memory. Although commonly accepted, this view has been challenged (LaBar and Disterhoft, 1998). The demands on neural resources increase with task complexity for both trace and delay conditioning (Knuttinen et al., 2001; Carter et al., 2003). Thus the differential requirement of the hippocampus for trace conditioning might be a result of task complexity and not of the discontinuity between stimulus and reinforcement (Carrillo et al., 2000; Beylin et al., 2001; Walker and Steinmetz, 2008; Kehoe et al., 2009). The necessity of awareness in trace conditioning was also challenged in recent studies which demonstrated that humans can also learn trace conditioning without awareness, such as when asleep (Arzi et al., 2012) or in a vegetative state (Bekinschtein et al., 2009). How delay and trace conditioning differ in their anatomical and physiological basis is still an open question? Where and how is the stimulus trace maintained until US arrival? Is the trace actively being kept in the brain or is it a passive decay of activity originated from the stimulus? What are the molecular correlates of the stimulus trace?

In recent years several approaches to study the cellular and molecular mechanisms of stimulus traces and trace memories have been performed in insects (Ito et al., 2008; Tomchik and Davis, 2009; Galili et al., 2011; Shuai et al., 2011; Szyszka et al., 2011), taking advantage of their ability to solve the trace conditioning task generally faster than vertebrates. Another beneficial aspect of using insects is based on their simpler brains which allow easier access for physiological measures and enable genetic and molecular manipulations. In this review, we summarize the findings regarding the behavioral, molecular, anatomical, and modeling data on trace conditioning in insects and point out the knowledge gaps that still wait to be filled.

SPECIAL AND COMMON FEATURES OF TRACE CONDITIONING

Insect trace conditioning has been mainly conducted in three behavioral paradigms. Early experiments were performed with freely flying honeybees, where the animals learned to associate a color which was presented during the approach of a food source, but not during feeding with a food reward. Successful conditioning was observed as a preference for the trained color during future landings (Opfinger, 1931; Menzel, 1968; Grossmann, 1970). Later studies were done in harnessed animals, pairing odor stimuli with a temporally separated sugar reward (*Apis mellifera*: Menzel, 1983; Szyszka et al., 2011; *Manduca sexta*: Ito et al., 2008). Associative memory formation was measured as proboscis extension reflex (PER) in response to the odor presented alone. In *Drosophila melanogaster*, aversive olfactory conditioning in the T-maze is the most commonly used paradigm for trace conditioning (Tully and Quinn, 1985; Tanimoto et al., 2004; Galili et al., 2011; Shuai et al., 2011). Here, a group of animals is trained to associate an odor with the following electric shock. During testing, animals have to choose between the previously punished odor and either a different odor or pure air.

The paradigm diversity may account for observed differences in learning performance when comparing trace with delay conditioning, within and between species. *Drosophila*, for example, is able to reach the same learning asymptote in olfactory delay and trace conditioning in the T-maze (Galili et al., 2011). In contrast, honeybees (*Apis mellifera*) did not reach the same learning asymptote in trace conditioning compared to delay conditioning, as measured with PER (up to 19 training trials in Menzel et al., 1993; up to six training trials in Szyszka et al., 2011). Further experiments are required to clarify if these disparities are paradigm-dependent effects.

In addition to these differences, shared features of delay and trace conditioning were also revealed, such as similar perception of odor identity and similar memory decay curves (Galili et al., 2011; Szyszka et al., 2011). Also the shape of CS–US interval functions showed resemblance across species and paradigms: **Figure 1B** shows a CS–US interval function in insects, which is strikingly similar to that of mammals (Rescorla, 1988); likewise,

the obtained curve for insect visual learning is similar to that for olfactory learning (Menzel, 1983).

One remarkable phenomenon in trace conditioning is the existence of paradigm learning. Previous trace conditioning improved pigeons' learning performance in subsequent trace conditioning with longer CS–US intervals (Lucas et al., 1981). The same effect was observable in insects. One-trial trace conditioning with a short CS–US interval enabled honeybees to succeed in initially unsolvable 1-trial trace conditioning with an extended CS–US interval (Szyszka et al., 2011). Similarly, *Drosophila* trained in five trials with increasing CS–US interval learned better than flies presented with the reverse order of intervals (Galili et al., 2011).

These studies show, that animals gain experience in trace conditioning which facilitates learning during subsequent trace conditioning. But what kind of experience is this? In honeybees, response latency during the test was shorter following training with delay conditioning (simultaneous CS and US onset) than with trace conditioning. Even if response latency did not correlate with the CS–US interval during trace conditioning (Szyszka et al., 2011), this result suggests that during training with trace conditioning animals learn something about US timing. This is in accordance with Pavlov (1927) who found a later conditioned response in dogs after trace conditioning.

What happens in the brain while the animal is waiting for the reinforcement? At the level of neuronal correlates, there are two possible ways how the previous successful performance in trace conditioning tasks can help bridging the longer gap in following trials. The first mechanism is prolongation of the CS trace until the arrival of the US, altering the CS representation pathway. Another possible mechanism is the activation of US-representing neurons during the CS (US anticipation), assuming that the animals learn a causal connection between the separated CS and US [as is the case in monkeys, reviewed by Schultz (2006)]. Since a single training trial is enough for some insects to learn, US anticipation can be excluded as a possible explanation for 1-trial trace learning. Nonetheless, after several trials, US anticipation may develop. The proposed mechanisms may also act together to improve consecutive trace conditioning trials. Behaviorally, US anticipation can enhance the CS saliency, so that the animal will assign greater importance to the fading CS trace by during consecutive trials.

An interesting characteristic of trace conditioning in vertebrates is that filling the gap with another stimulus enhances learning (Kamin, 1965; Kaplan, 1984), whereas interference is detrimental to non-associative short-term memories like habituation and sensitization.

This learning enhancement was also shown in olfactory trace conditioning in honeybees, where a second CS within the gap strengthened the association between the first CS and the US (Szyszka et al., 2011). How does an additional stimulus during the gap improve trace conditioning? It might act as a distinguishing feature from the background, which changes the environmental context, creating a "bridge" between CS and US. In a natural environment where stimuli follow each other with varying time intervals, such a distinguishing feature may help the animal to resolve the temporal ambiguity between the CS and US, i.e., whether a US is related to a preceding CS, or to a following CS (Beylin et al., 2001).

WHEN IS THE ODOR TRACE INITIATED?

Depending on the stimulus length, both the onset and the offset of a stimulus can serve as a CS (Kehoe et al., 2009). This was found in rabbit nictitating membrane trace conditioning (where a tone is associated with an air puff to the eye) when a CS of several hundred milliseconds or longer was used (Desmond and Moore, 1991; Kehoe and Weidemann, 1999; Kehoe and Macrae, 2002).

Behavioral studies in honeybees (Szyszka et al., 2011) and *Manduca sexta* (Ito et al., 2008) indicated that the initial part of an odor stimulus and not the late phase or odor offset triggers the stimulus trace, whereas in *Drosophila* the odor offset seems to elicit a trace (Galili et al., 2011). The different time points of trace initiation might explain why the CS–US interval learned by *Drosophila* (CS–US interval: 25 s; gap: 15 s; Galili et al., 2011) was longer than that learned by honeybees (CS–US interval: 6 s; gap: 5.5 s; Szyszka et al., 2011). The observed behavioral differences might indeed be a matter of stimulus length (Kehoe et al., 2009), which varied between 0.5 s in the honeybee study to 10 s in the *Drosophila* study.

Ecologically, the observed differences in trace initiation might account for species-specific requirements. Fast flying insects such as honeybees and moths possess a remarkable ability to identify and track a single odor in a highly turbulent, multi-odor background. This ability relies on analyzing and remembering the temporal structure of odor plumes, which contain information about the distance and location of the odor source (Vickers, 2000; Cardé and Willis, 2008). Therefore, fast flying insects may need to stay receptive for new odors which they might encounter during flight. They may need to remember when they encounter an odor plume rather than when they leave it. In contrast, slow flying insects, such as *Drosophila*, live in a more static olfactory environment. Living in such a habitat, it might be more important to be sensitive to concentration gradients rather than to on- and offsets of fast fluctuating odors. Altogether, species-specific differences have to be considered when searching for the neural correlates of CS traces and CS–US association during trace conditioning.

Finally, different methods of odor delivery in the behavioral studies [automatic in Szyszka et al. (2011) vs. manual in Galili et al. (2011)] may account for the observed differences in trace initiation between honeybees and *Drosophila*. These behavioral studies indicated the time windows in which the stimulus traces in different species are triggered. This information is crucial to focus on the particular time window in physiological experiments aiming at the identification of stimulus traces.

NO EVIDENCE YET FOR TRACE-RELATED NEURAL ACTIVITY

Where is the information about the CS stored until the US arrives? The insect olfactory pathway (**Figure 2**) starts at the antennae where odors activate odor-specific subsets of olfactory receptor neurons (ORNs). ORNs transmit odor information to the antennal lobe, the primary brain area for olfactory processing. Here, ORN axons interact with excitatory and inhibitory local interneurons (LNs) and with projection neurons (PNs) which conduct the information to higher order neurons, like the Kenyon cells (KCs)

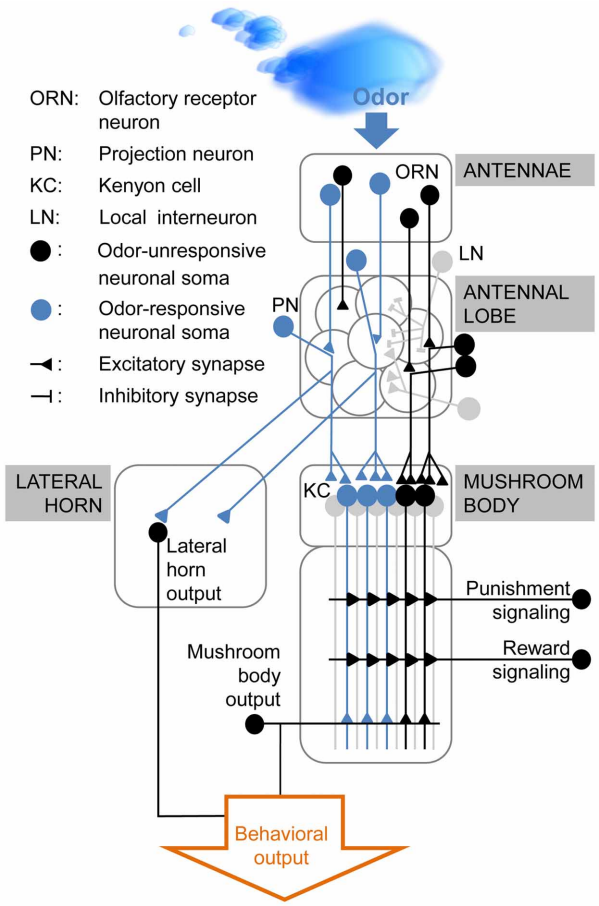

FIGURE 2 | Simplified diagram of olfactory processing in *Drosophila melanogaster*. Odors activate distinct sets of olfactory receptor neurons (ORNs) in the fly's antenna. ORNs which express the same receptor converge onto the same glomerulus in the antennal lobe. Thus, each odor induces a unique glomerular activity pattern. This pattern is modulated by the interaction between ORNs, excitatory and inhibitory local interneurons (LNs) and projection neurons (PNs). The PNs relay the odor information to the lateral horn (which is assumed to mediate the innate odor response) and to the mushroom body intrinsic Kenyon cells (KCs). Upon reinforcement, KCs receive input from modulatory neurons, signaling either punishment or reward. Coincident activation of these neurons and the odor-responsive KCs in delay conditioning is believed to modify the output in these KCs. This modification changes the fly's behavior to the previously reinforced odor stimulus. For trace conditioning the underlying mechanism is yet unknown, but it seems likely that the necessary modifications occur in the olfactory pathway and/or pathway-associated neurons.

in the mushroom body and lateral horn neurons (**Figure 2**). The olfactory trace might be located in any of these neuron types or in other neurons outside the olfactory pathway.

There is good evidence that odor identity is encoded in antennal lobe odor response patterns. The perceived odor similarity (extracted from behavioral odor generalization experiments) corresponds to the physiological odor similarity (as measured with calcium imaging, comparing odor-evoked combinatorial glomerular activity patterns) during odor presentation. This was shown in the honeybee (Guerrieri et al., 2005) and *Drosophila* (Niewalda et al., 2011).

If ORNs or PNs encode the trace, their physiological post-odor similarity profile should follow the same correlation as the behavioral similarity profile and predict the perceived similarity profile during trace conditioning. However, such correlation between physiological post-odor activity and behavioral generalization after trace conditioning was neither found in *Drosophila* ORNs (Galili et al., 2011) nor in honeybee PNs (Szyszka et al., 2011). These findings indicate that the odor trace should be located downstream of the PNs. Consistently, analyzing action potential firing patterns in honeybee PNs, Nawrot (2012) found that the correlation between the initial and later phases of odor response patterns was high, but rapidly decreased with odor offset (Krofczik et al., 2008). Similarly, post-odor activity in mouse mitral cells is odor specific but different from the odor response (Bathellier et al., 2008). The common finding of specific post-odor activity in different cell populations in the olfactory pathway suggests that this feature, though it does not correlate directly with trace conditioning, may be an evolutionarily conserved property. It remains to be shown whether such post-odor response patterns have functional relevance or whether they are a mere byproduct of odor processing.

From these findings it can be concluded that an olfactory stimulus trace does not consist of persistent neuronal activity in ORNs or PNs. But these findings do not rule out the possibility that the stimulus trace is encoded in antennal lobe LNs, or in any of these neuron types as biochemical modifications (Perisse and Waddell, 2011). Subtle network activity, such as changes in the correlation of glomerular spontaneous activity after the presentation of an odor (Galan et al., 2006) might also be an underlying mechanism.

A more promising brain structure, however, is the mushroom body which is the site where different stimulus modalities converge and associative olfactory learning occurs (Erber et al., 1980; Heisenberg et al., 1985; Menzel, 2001). Consistently, Shuai et al. (2011) suggested a role of *Drosophila* KCs in trace conditioning, based on studies with Rac which is a small G protein belonging to the Rho family of GTPases. Elevated Rac activity in the mushroom bodies was shown to accelerate memory decay after olfactory aversive delay conditioning (Shuai et al., 2010). In trace conditioning, the targeted inhibition of Rac in the mushroom bodies but not in the antennal lobes increased the trace-dependent memory formation. Furthermore, rescue experiments in dopamine (DA) receptor mutants showed that D1 DA receptor expression in mushroom bodies was required for trace conditioning (Shuai et al., 2011).

POST-ODOR RESPONSES IN KENYON CELLS

KCs have the intriguing property of responding mainly to odor onset, less to odor offset and even less to ongoing odor stimulation (*Schistocerca americana*: Perez-Orive et al., 2002; *Apis mellifera*: Szyszka et al., 2005; *Manduca sexta*: Ito et al., 2008; *Drosophila melanogaster*: Murthy et al., 2008; Turner et al., 2008).

In *Manduca sexta*, the probability of KC offset responses increased with stimulus length (Ito et al., 2008), though offset responses were elicited in a different set of KCs than the onset responses (Ito et al., 2008). Reinforcement of the CS offset in behavioral experiments did not result in learning (Ito et al., 2008). But this might be a species or paradigm-specific observation.

KC offset responses in other species have not been examined with respect to their involvement in trace conditioning to date. Indeed, the evidence for an involvement of KCs in trace conditioning (Shuai et al., 2011) and their accepted role in delay conditioning require further investigation of KC post-stimulus activities and their possible trace encoding properties. The trace might be encoded as biochemical tagging of odor-encoding KCs (Wessnitzer et al., 2012). In addition, odor-responsive KCs might become reactivated during the pairing of an odor with a US (Szyszka et al., 2008) and this reactivated KC ensemble might encode the trace (Szyszka et al., 2011).

In vertebrates, delay and trace conditioning rely on different brain structures. Such a distinction on the circuit level might also be true for insects. Exemplified in *Drosophila*, the trace might be encoded by LNs in the antennal lobe (**Figure 2**), by modulatory neurons like the anterior paired lateral neuron (APL; Liu and Davis, 2009) or the dorsal paired medial neuron (DPM; Waddell et al., 2000), as suggested by Perisse and Waddell (2011) or by other mushroom body extrinsic neurons (Tanaka et al., 2008).

MOLECULAR REQUIREMENTS OF LEARNING DURING TRACE AND DELAY CONDITIONING

Behavioral studies revealed not only differences between delay and trace conditioning, but also many similarities, which suggest that the transition between these two forms of learning might be continuous (Menzel, 1983; Tully and Quinn, 1985; Tanimoto et al., 2004; Galili et al., 2011; Szyszka et al., 2011). But do these similarities originate from related molecular mechanisms?

Recently, genetic and physiological studies in *Drosophila* gave insights into the molecular requirements of both conditioning forms (Tomchik and Davis, 2009; Shuai et al., 2011). Trace conditioning does not involve the Rutabaga adenylyl cyclase (Rut-AC; **Figure 3A**; Shuai et al., 2011), which is required for delay conditioning (Duerr and Quinn, 1982; Dudai et al., 1983). Furthermore, the inhibition of Rac enhanced learning performance in *Drosophila* trace conditioning, while delay conditioning remained unaffected (Shuai et al., 2011).

Do these differences derive from the necessity to bridge the temporal gap or from differences in task complexity? In most cases, trace conditioning yields lower memory performance than delay conditioning. Thus weak learning paradigms in general may recruit alternative molecular pathways compared to paradigms which induce strong learning, as suggested in mammal studies (Beylin et al., 2001). In support of this idea, Rac inhibition also enhanced the learning performance in delay conditioning, when it was performed with low odor concentrations, normally leading to low memory performance (Shuai et al., 2011, Supplementals).

Tomchik and Davis (2009) investigated the role of cyclic adenosine monophosphate (cAMP) signaling in delay and trace conditioning. The authors pharmacologically simulated the CS and US by application of acetylcholine (ACh) and DA or octopamine (OA), respectively, onto dissected *Drosophila* brains (**Figure 3A**). The CS–US timing was chosen according to standard conditioning protocols. The cAMP increase was synergistic for paired ACh–DA applications, compared to the summed response of unpaired applications. This synergistic effect was observed for ACh–DA pairings in both, a delay and a trace conditioning manner (for an ACh–DA interval $\leq$15 s). The approach revealed no differences between the delay and trace conditioning simulations.

In the delay conditioning simulation the synergistic cAMP increase was Rut-AC dependent, while Rut-AC dependency was not tested in the trace conditioning simulation. According to Shuai et al. (2011) trace conditioning is Rut-AC independent and the observed synergistic cAMP increase (Tomchik and Davis, 2009) thus might be induced by another coincidence detector. Pairing ACh with OA application in a delay conditioning manner resulted in a subadditive effect, which was Rut-AC independent. Simulation of trace conditioning was not tested with OA. These results provide hints for a role of cAMP in trace conditioning (**Figure 3A**), although it has to be taken into account that the duration of the ACh and DA bath applications is not as precisely controllable as stimuli in behavioral paradigms.

SEARCHING FOR THE COINCIDENCE DETECTOR IN TRACE CONDITIONING

As Rut-AC may not be involved in olfactory trace conditioning in *Drosophila* (Shuai et al., 2011), other coincidence detectors may account for the CS–US association. One candidate is the N-methyl-D-aspartate-type glutamate (NMDA) receptor (Traynelis et al., 2010; Miyashita et al., 2012). The Mg^{2+} block of this receptor plays an important role in insect olfactory learning (Miyashita et al., 2012). Only upon correlated activity of a presynaptic and a postsynaptic cell, Mg^{2+} is removed and the channel opens. The resulting large Ca^{2+} influx is crucial for learning (**Figure 3B**).

From studies in mammals it is known that in some instances NMDA receptors play a role in trace conditioning, but not in delay conditioning. Blocking of NMDA receptor-mediated signaling in the prefrontal cortex of rats modified gene expression pathways in the hippocampus and impaired trace, but not delay fear conditioning (Gilmartin and Helmstetter, 2010; Czerniawski et al., 2012). Could it be that NMDA receptors, in a similar fashion, act as coincidence detectors in insect trace conditioning?

The *radish* gene encodes a protein that is highly expressed in the MBs (Folkers et al., 2006) and was suggested to be involved in Rut-AC independent delay conditioning (**Figure 3B**; Isabel et al., 2004). Is it possible that Rut-AC independent trace conditioning relies on Radish function as well?

Another candidate for coincidence detection is Gilgamesh (Gish), a casein kinase Iγ homolog. Gish is required for Rut-AC independent olfactory learning in *Drosophila* (Tan et al., 2010) and it accounted for the residual delay learning in Rut-AC and protein kinase A (PKA) mutants (**Figure 3B**; Skoulakis et al., 1993; Han et al., 2003). Whether and how Gish functions as a coincidence detector is unknown. Gish is supposed to mediate intracellular Ca^{2+} increase in those MB neurons which respond to the reinforced CS (Tan et al., 2010). The study by Tan et al. (2010) showed that delay conditioning is achieved via separate pathways (either Rut-AC or Gish-dependent). Further studies are needed to answer the question of whether the Radish- and/or Gish-dependent pathways are shared in olfactory trace and delay conditioning.

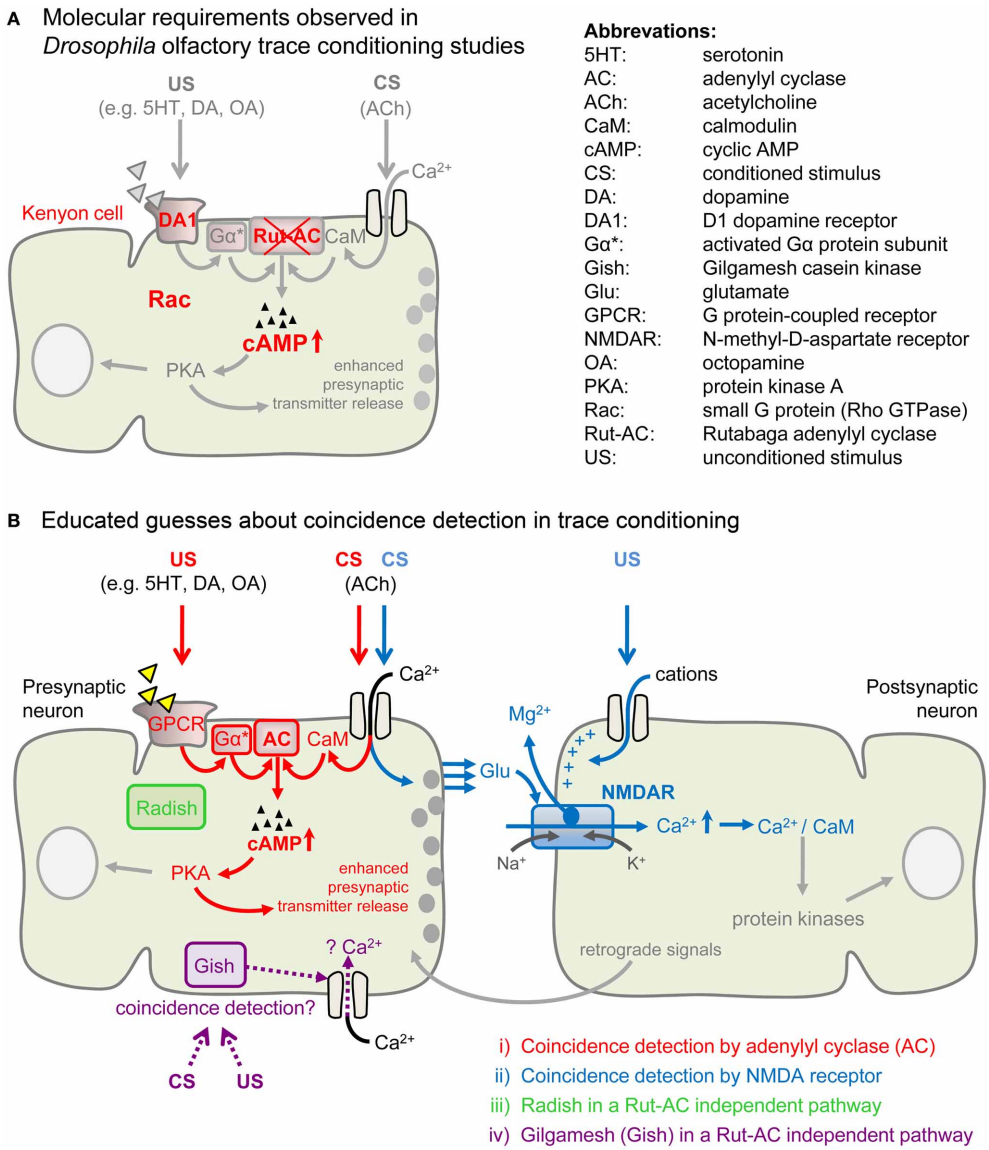

FIGURE 3 | Molecular requirements for trace conditioning and four non-exclusive models of possible coincidence detection. (A) Cellular and molecular requirements which were shown to contribute to *Drosophila* olfactory trace conditioning. Shuai et al. (2011) found that the targeted inhibition of Rac in the mushroom bodies increased trace-dependent memory formation. Also D1 dopamine receptor (DA1) expression in mushroom bodies was required for trace conditioning, as shown by rescue experiments (Shuai et al., 2011). Trace conditioning does not require the Rutabaga adenylyl cyclase (Rut-AC; Shuai et al., 2011), but delay and trace conditioning simulations both induced synergistic increases of cAMP (Tomchik and Davis, 2009). **(B)** Educated guesses about coincidence detection in delay conditioning might also apply to trace conditioning. **(i)** Presynaptic coincidence detection by an adenylyl cyclase (AC; shown in red). In the presynaptic neuron, the CS induces Ca^{2+} influx and Ca^{2+} binds to calmodulin (CaM). The US activates G protein-coupled monoaminergic receptors (GPCR) which activate the associated G protein (Gα). When Ca^{2+}/CaM complex and activated G protein (Gα*) co-occur, the AC is activated more strongly than if they appear alone. This leads to an increased production of cAMP and to activation of protein kinase A (PKA) which enhances presynaptic transmitter release (Heisenberg, 2003). **(ii)** Postsynaptic coincidence detection by the N-methyl-D-aspartate-type glutamate (NMDA) receptor (shown in blue). The CS leads to presynaptic release of glutamate (Glu) which binds to the postsynaptic NMDA receptor. The US, on the other hand, induces the depolarization of the postsynaptic membrane, which allows for the removal of the Mg^{2+} block from the NMDA receptor channel. Opening of the NMDA receptor channel for Ca^{2+} influx is only possible when the CS and the US signal coincide. An elevation of the intracellular Ca^{2+} level leads to the activation of several kinases, inducing synaptic plasticity. The NMDA receptor is involved in delay conditioning in *Drosophila* (Miyashita et al., 2012) and was also shown to be involved in trace conditioning in vertebrates (Gilmartin and Helmstetter, 2010; Czerniawski et al., 2012). A possible role in insect trace conditioning has not yet been investigated. **(iii)** Radish (shown in green) is involved in a Rut-AC independent pathway (Isabel et al., 2004; Folkers et al., 2006) and might contribute to trace conditioning. **(iv)** Gilgamesh (Gish) (shown in purple), a casein kinase I γ homolog in flies, is required for short-term memory formation in *Drosophila* olfactory delay conditioning, functioning independently of Rut-AC and the cAMP pathway (Tan et al., 2010). Hypothetically, Gish mediates increased Ca^{2+} influx upon CS–US coincidence and thus might be a pathway for Rut-AC independent trace conditioning.

Recent studies in rats have revealed a role for serotonin in mammalian trace conditioning (Miyazaki et al., 2011). The activity of serotonin neurons was increased when rats had to wait for a delayed reward. Serotonin has not yet been tested in insect trace conditioning, and—together with other possible neuromodulators—may be a promising target for future studies.

COMPUTATIONAL MODELS REVEAL POTENTIAL MECHANISMS FOR TRACE LEARNING

Computational modeling further supports the intriguing search for the underlying mechanisms of trace conditioning. Several modeling approaches aiming at the neural circuits and/or molecular mechanisms of associative learning might help to understand trace conditioning (Desmond and Moore, 1988; Drew and Abbott, 2006; Izhikevich, 2007; Yarali et al., 2012). The models are based on the mechanism of synaptic plasticity: strengthening the synapses where stimuli coincide.

A process accounting for association on millisecond timescale is spike timing dependent plasticity (STDP) which is involved in both long term potentiation and long term depression of synapses. A synapse is strengthened and synaptic transmission is increased when presynaptic action potential firing precedes postsynaptic firing within a short time window of a few milliseconds. The reverse order weakens the synapse and reduces synaptic transmission.

In associative conditioning, pre- and postsynaptic firing induced by CS and US, respectively, would result in synaptic strengthening. When the CS is presented alone after many pre–post pairings, the post-neuron might fire without a US input. This strengthened synaptic connection reflects associative learning.

However, there is a timescale discrepancy regarding stimulus timing in behavior and STDP (reviewed in: Gallistel and Matzel, 2013). On the behavioral level, actions often elapse over several seconds, while the physiological timescale of STDP extends only over milliseconds. In delay conditioning the CS spikes could overlap with the US spikes and thus lead to potentiation of those synapses. In trace conditioning, this coincidence would not be possible since the CS and the US are several seconds apart.

To account for this discrepancy, Drew and Abbott (2006) assumed in their model that a CS evokes in the presynaptic neurons long spike trains of action potentials with slowly decaying spike rates after stimulus offset. The residual spiking serves as a trace and can coincide with the postsynaptic US spiking, increasing the synaptic strength. In this model, repeated pairing of CS–US led to potentiation of the synaptic efficacy, enabling postsynaptic firing from presynaptic activation alone. The incorporation of slow firing rate decays into the STDP model solved the observed timing problem for trace conditioning. However, the key assumption of this model (that long spike trains follow stimulus termination) contradicts with the physiological findings in olfactory learning. The KCs, which are assumed to be the site of CS–US coincidence, do not evoke such long spike trains, but only very sparse and short-lasting responses upon odor application (Szyszka et al., 2005; Ito et al., 2008).

Other models suggested that the combination of STDP and neuromodulators might solve the timescale discrepancy and explain coincidence detection in trace conditioning. Izhikevich (2007) suggested a network where transient synaptic changes, induced by coincident pre- and postsynaptic spiking (following the STDP rule), were enhanced by a DA reinforcement (**Figure 4Ai**). These transient synaptic changes—acting as synaptic eligibility traces—could be the activation of an enzyme with slow kinetics, important for synaptic plasticity. In the model, these eligibility traces were exponentially decaying over several seconds. During this decay, the synapse got reinforced by a global DA release (1–3 s after the STDP; **Figure 4Ai**) leading to a reinforcement of the synaptic eligibility trace and strengthening of the synapse. Other synapses in the network that also elicited coincident firing which was not linked to the reward, were not strengthened. Repetition of reinforcing each such pre–post firing event increasingly strengthened the particular synapse. This in turn increased the probability of coincident firings at this synapse, leading to even more reinforcement (**Figure 4Aii**). The model shows how STDP might also contribute to insect trace learning when the fast STDP mechanism is combined with slower biochemical processes and subsequently mediated by neuromodulators.

This idea was experimentally tested in the mushroom bodies of locusts. Cassenaer and Laurent (2012) examined the effect of neuromodulators (specifically OA) on the plasticity of KC output synapses onto their postsynaptic targets, the beta-lobe neurons. The synapses at which pre- and postsynaptic action potentials were coinciding seemed to be tagged, and only the tagged synapses were subsequently modified by OA, which was applied 1 s after the STDP. This process could underlie delay and also trace conditioning as the temporal gap in trace conditioning might be bridged by the synaptic eligibility trace (Izhikevich, 2007), and specific synapses would then be reinforced by the neuromodulator. With respect to trace conditioning, it would be interesting to know if gaps longer than 1 s between the STDP and the application of neuromodulators have an effect on synaptic plasticity, and whether gap length and corresponding synaptic plasticity fit to behavioral observations.

In addition to STDP, other mechanisms have been proposed to account for associative learning. The model by Yarali et al. (2012) refers to aversive olfactory learning in *Drosophila melanogaster* and is based on the mechanism of coincidence detection by an adenylyl cyclase (AC). It suggests that slowly decaying Ca^{2+} transient in the presynaptic neuron, elicited by the CS, could function as a stimulus trace. The odor-induced Ca^{2+} signal (Wang et al., 2004; Yu et al., 2006; Wang et al., 2008; Honegger et al., 2011) and the shock-induced DA signal (Schwaerzel et al., 2003; Riemensperger et al., 2005; Kim et al., 2007; Claridge-Chang et al., 2009; Aso et al., 2010) converge in the mushroom body KCs, where they synergistically activate an AC (**Figure 4Bi**). The activation of the AC by the US signal (via an activated G protein subunit, Gα*) is bidirectionally modulated by the CS-induced Ca^{2+} influx depending on the relative timing of the CS and the US (**Figure 4Bii**). The Ca^{2+} influx transiently increases the rate constants for both the formation and the dissociation (k_A and k_D, **Figure 4Bii**) of the active AC*/Gα* complex.

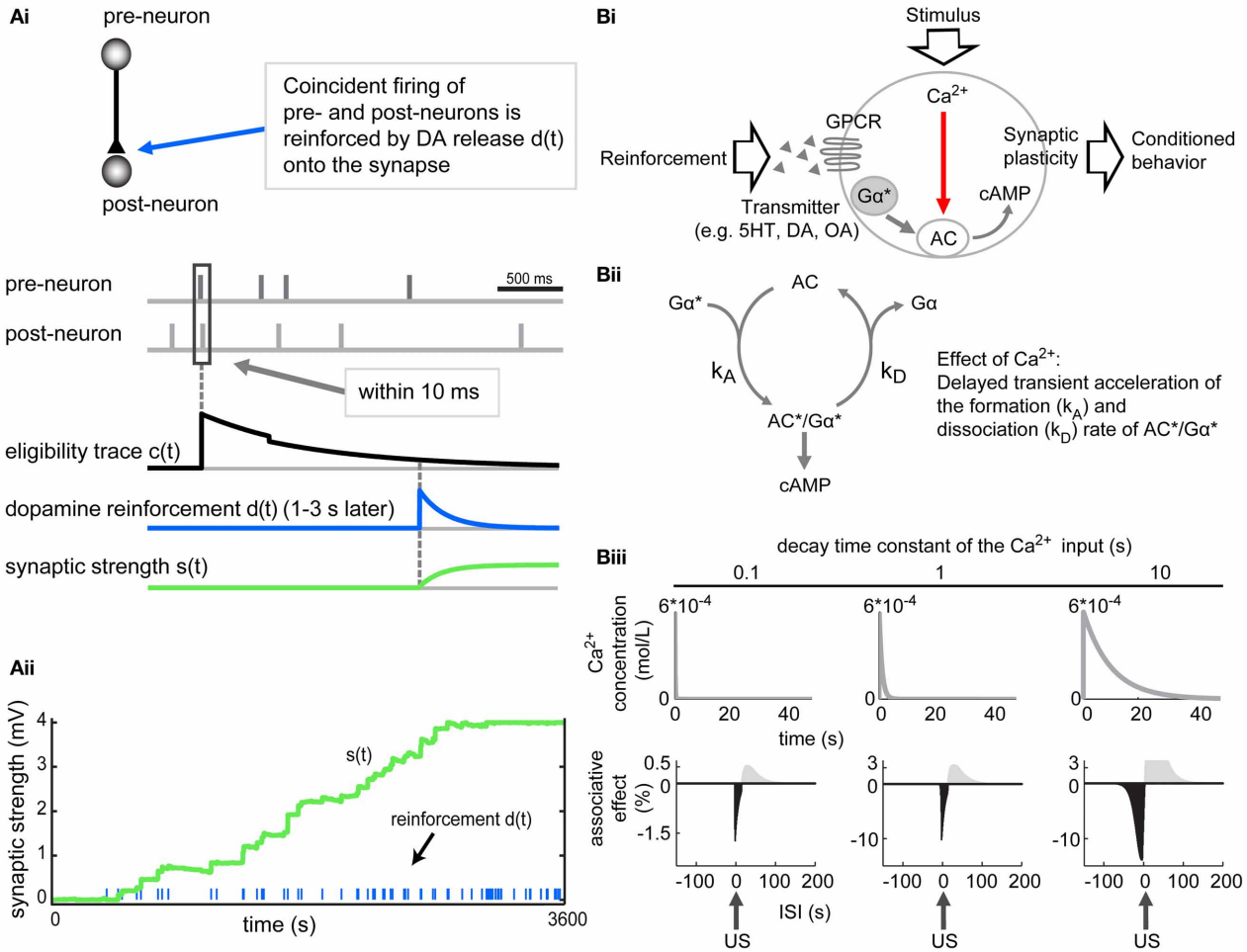

FIGURE 4 | Models relying on spike timing dependent plasticity (STDP) or biochemical processes can account for trace processes. **(Ai)** In the model by Izhikevich (2007), the coincident firing of a pre- and then a postsynaptic neuron (within 10 ms; marked by a rectangle) elicits a synaptic eligibility trace c(t) in the corresponding synapse. This eligibility trace decays exponentially to zero. Reinforcement d(t), here a dopamine (DA) release delayed by 1–3 s in combination with the residual eligibility trace, increases the synaptic strength s(t) [s(t) = c × d] of the particular synapse. **(Aii)** Repeated reinforcement of such a pre–post firing event increasingly strengthens the particular synapse. This in turn increases the probability of coincident firings of this synapse. Adapted from Izhikevich (2007), with permission. **(B)** Lingering Ca^{2+} and coincidence detection by an adenylyl cyclase (AC) might account for trace conditioning in the model by Yarali et al. (2012). **(Bi)** Ca^{2+} influx and Gα activation (induced by CS and US, respectively) synergistically act on the AC, leading to increased cAMP production and strengthening of the synaptic output. **(Bii)** In this model Ca^{2+} is supposed to transiently accelerate both the formation and dissociation rates (k_A and k_D) of the AC*/Gα* complex to the same extent. When the system is in equilibrium (k_A and k_D are the same), Ca^{2+} has no effect on the cAMP level. But when Ca^{2+} influx shortly precedes the transmitter induced activation of Gα*, the formation (k_A) of AC*/Gα* is at this time point the dominant reaction. This leads to a rise in AC*/Gα* concentration and thus, enhanced cAMP production. When Ca^{2+} influx follows Gα*, the dissociation of AC*/Gα* is promoted, leading to decreased cAMP production. **(Biii)** This model can account for trace conditioning by changing the Ca^{2+} decay time constants (different decay time constants chosen are 0.1, 1 and 10 s). The larger decay constants (e.g., 10 s) cause a long tail of Ca^{2+} transient (upper row). This allows for associations of stimuli over longer interstimulus intervals (ISIs; bottom row) and is critical for reproducing the behavioral measurements of trace conditioning. The longer the Ca^{2+} decay time is, the larger the negative "associative" effect is in the simulation. This reveals that lingering Ca^{2+} in KCs might contribute to bridge the temporal gap between CS and US. Note that in this model the US onset is set to 0 and the CS onset shifts to the left for increasing ISIs (CS–US intervals). The negative associative effects correspond to the learned odor avoidance in olfactory aversive delay and trace conditioning. The Ca^{2+} influx is always constant (rising to a Ca^{2+} concentration peak of 6×10^{-4} mol/L within 40 ms). Adapted from Yarali et al. (2012).

Based on this mechanism of coincidence detection by the AC, odor-shock conditioning in *Drosophila* was simulated. When the odor-induced Ca^{2+} influx shortly preceded the US-induced G protein activation (Gα*) as in delay conditioning, the formation of the AC*/Gα* complex was transiently accelerated. This led to increased cAMP production resulting in potentiation of synaptic output in these particular KCs. In trace conditioning where the CS is already gone upon US arrival, the coincidence could be achieved by residual Ca^{2+} transient in the cell.

To test if the model is capable of predicting trace conditioning, the authors changed the shape of the Ca^{2+} signal such that at the moment of US arrival, there was still sufficient Ca^{2+} present

to induce plasticity (**Figure 4Biii**). This residual Ca^{2+} was critical for reproducing the behavioral measurements of trace conditioning. The slower the simulated decay of Ca^{2+} was the larger was the "associative" effect in the simulation (**Figure 4Biii**). Thus lingering Ca^{2+} in KCs could contribute to bridge the temporal gap between two stimuli. In *in vivo* studies long-lasting Ca^{2+} concentration in KCs was neither confirmed nor excluded (Wang et al., 2004; Yu et al., 2006; Wang et al., 2008).

This model (Yarali et al., 2012) gives a simple biochemical explanation for delay and trace conditioning based on the modulation of AC activation by the transient Ca^{2+} level. The components of this model, namely the cAMP formation by the AC have been experimentally investigated by Tomchik and Davis (2009). Synergistic increase of cAMP in α and α′ lobes of the mushroom bodies was induced by pharmacologically mimicking CS and US in dissected *Drosophila* brains. Moreover, the cAMP pathway itself was shown to be strongly involved in learning (Gervasi et al., 2010).

Note that some of the described models cannot account for 1-trial trace conditioning since they are based on repeated stimulus pairings.

METHODOLOGICAL CONSIDERATIONS

The variety of trace conditioning paradigms renders a comparison of the obtained results rather difficult. Each method has its own peculiarity, such as the properties of the chosen CS or US. According to the Rescorla and Wagner model for classical conditioning (Rescorla and Wagner, 1972), learning directly depends on the salience and intensity of the CS and the US. Given that trace conditioning in most cases is less efficient than delay conditioning, this difference can be explained by a reduced CS salience in trace conditioning. The CS salience probably decays until the US is applied. Not only does the length of the CS–US interval have considerable impact on the CS salience, but so does the CS identity (Pavlov, 1927). Thus, trace conditioning studies using different CS are not necessarily comparable.

Some CS modalities hold potential pitfalls, as shown for the very common olfactory trace conditioning paradigms. We found that many odors are "sticky" and linger in the training device (Galili et al., 2011), such that it is impossible to clearly distinguish between trace and delay conditioning. Therefore, proper controls are important to exclude residual odor in the training device, e.g., behavioral controls such as unpaired stimulus presentation (Galili et al., 2011), physiological controls such as calcium imaging from olfactory neurons (Szyszka et al., 2011) or technical controls such as photoionization measurements (Shuai et al., 2011).

What other kinds of stimulus modalities seem suitable for trace conditioning? There are several studies indicating that visual stimuli are promising. To our knowledge the first report about visual trace conditioning in insects is from the early 1930s. Opfinger (1931) demonstrated that the color presented during the approach of a food source is learned better by honeybees than the color presented during feeding. *Drosophila* are also able to remember visual stimuli. They can remember the position of a vanished visual object and use this information for navigation (Neuser et al., 2008). In the past 50 years, several visual trace conditioning studies have been carried out (Menzel, 1968; Grossmann, 1970, 1971; Menzel and Bitterman, 1983) showing that visual stimuli are well suited to study this learning form.

The sensory pathways underlying trace conditioning certainly depend on the stimulus modality. However, the shape of the CS–US interval function in visual and olfactory conditioning looks very similar (Menzel and Bitterman, 1983). Thus the cellular mechanisms for keeping the CS trace may be related in different modalities. It is also evident that the suitability of the US for conditioning paradigms depends on the responsiveness of the animal (Pavlov, 1927).

CONCLUSIONS

In this review we described recent findings regarding the behavioral, molecular, physiological, and modeling aspects of insect trace conditioning. We noted some differences in the features of trace conditioning between different studies. For instance, in bees the initial part of a stimulus initiates the stimulus trace whereas it seems to be the end of a stimulus that initiates the trace in *Drosophila*. In bees and *Drosophila*, trace conditioning seems to yield lower memory performance than delay conditioning paradigms, whereas it is the opposite in *Manduca sexta*. Whether these are species-specific differences caused by adaptation to diverse natural habitats or paradigm-dependent differences remains to be shown. Comparing trace conditioning between similar paradigms in different species and between different paradigms in the same species might give the answer. We also highlighted many common properties of trace conditioning. One example is the commonly shared shape of CS–US interval function across species and paradigms. Such communalities make us believe that an integrative approach will be auspicious for revealing the fundamental mechanisms behind trace conditioning. Insects are perfectly suited for such a comparison because they learn quickly, and they allow for a rich repertoire of conditioning paradigms. These include, among others, appetitive olfactory conditioning in honeybees (Matsumoto et al., 2012; Menzel, 2012), bumble bees (Riveros and Gronenberg, 2009), *Drosophila* (Tempel et al., 1983; Chabaud et al., 2006), ants (Guerrieri and d'Ettorre, 2010), *Manduca sexta* (Ito et al., 2008) and locusts (Simoes et al., 2011), aversive olfactory conditioning in *Drosophila* (Tully and Quinn, 1985) and honeybees (Abramson, 1986; Vergoz et al., 2007), visual conditioning in honeybees (Dobrin and Fahrbach, 2012), and auditory conditioning in *Drosophila* (Menda et al., 2011).

Salience of a CS and US influence the strength of associative memories (Rescorla and Wagner, 1972). Compared to delay conditioning, the generally lower performance in trace conditioning could reflect a lower salience of the CS and/or US. It will therefore be interesting to study how the salience of both the CS and the US influences learning and memory in trace conditioning. Could one reach the same stimulus salience and thus equal acquisition and memory performance in trace and delay conditioning?

In this review, we discussed alternative mechanisms that may account for trace conditioning, such as recurrent neuronal firing, residual Ca^{2+} transients, slowly decaying eligibility traces and different coincidence detectors apart from the well-studied Rut-AC. We are still far away from understanding how stimulus traces are encoded

in the brain and how the coincidence detection between a stimulus trace and the US is achieved. Do trace and delay conditioning in insects engage different neural circuits, as is the case in vertebrates? *Drosophila*, with the possibility to genetically manipulate identifiable neurons, appears to us as the most promising model, as it allows a truly integrative approach to address these questions from molecular to circuit level.

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Behavioral and neural plasticity caused by early social experiences: the case of the honeybee

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Cognitive experiences during the early stages of life play an important role in shaping future behavior. Behavioral and neural long-term changes after early sensory and associative experiences have been recently reported in the honeybee. This invertebrate is an excellent model for assessing the role of precocious experiences on later behavior due to its extraordinarily tuned division of labor based on age polyethism. These studies are mainly focused on the role and importance of experiences occurred during the first days of the adult lifespan, their impact on foraging decisions, and their contribution to coordinate food gathering. Odor-rewarded experiences during the first days of honeybee adulthood alter the responsiveness to sucrose, making young hive bees more sensitive to assess gustatory features about the nectar brought back to the hive and affecting the dynamic of the food transfers and the propagation of food-related information within the colony. Early olfactory experiences lead to stable and long-term associative memories that can be successfully recalled after many days, even at foraging ages. Also they improve memorizing of new associative learning events later in life. The establishment of early memories promotes stable reorganization of the olfactory circuits inducing structural and functional changes in the antennal lobe (AL). Early rewarded experiences have relevant consequences at the social level too, biasing dance and trophallaxis partner choice and affecting recruitment. Here, we revised recent results in bees' physiology, behavior, and sociobiology to depict how the early experiences affect their cognition abilities and neural-related circuits.

Keywords: behavior, honeybee, olfaction, plasticity, early experiences, associative learning

FRAMEWORK

Experiences at early stages of animals' life can shape later behavior in a dramatically and sometimes irreversible way. From the most extreme example of imprinting describing the attachment behaviors of geese soon after they hatched (Lorenz, 1935), a great range of sensory and cognitive experiences have been reported to play a role in shaping future behavior in many groups, including humans (Neal, 1972; Cornwell-Jones et al., 1988; Cramer et al., 1988; Gschanes et al., 1998; Matthews and Robbins, 2003; Pryce and Feldon, 2003; Schäble et al., 2007). In the last three decades, the honeybee *Apis mellifera* has been considered a model within the invertebrates to study the behavioral and neural plasticity caused by early experiences (Masson and Arnold, 1984, 1987; Winnington et al., 1996; Sigg et al., 1997; Farris et al., 2001; Brown et al., 2004). There are several reasons to justify this choice. First, honeybees exhibit an excellent predisposition to learn and retain neutral stimuli. Second, they have a relatively simple and accessible brain. Third, it is possible to manipulate the early experience of honeybees by assessing responses under controlled conditions. Finally and probably the most important reason is the changing behavioral contexts at which they are exposed during the adult lifespan.

Honeybees undergo an age-related polyethism which plays an important role in task allocation and division of labor within their colonies (Wilson, 1971; Michener, 1974). Newly emerged bees of the worker caste mainly clean the comb cells and care for brood inside the nest, while middle age bees process and store food until they initiate foraging from the third week of the adult life (Rösch, 1925; Lindauer, 1952; Seeley, 1982). This dynamic makes honeybees ideal models to analyze the effect of particular sensory stimuli during young adulthood on later behavior. Division of labor occurs because individuals differ in their preferred response. In a honeybee hive, nest-mates of different ages display different response thresholds to given stimuli, which enable the display of specific behaviors (Robinson, 1992; Page et al., 1998). The transition from one task to the next requires major changes in sensory and cognitive abilities which are accompanied by morphological and physiological changes in the brain (Fahrbach, 2006). Shift from in-hive tasks to foraging involves the development of a series of new and integrated skills such as flight navigation, food location and communication of information to the rest of the colony. At foraging ages the neural pathways are fully developed (Masson and Arnold, 1987; Winnington et al., 1996), providing bees with abilities to learn and retain relevant environmental stimuli. Foraging bees can, for example, learn floral odors while visiting rewarding flowers. Learning of olfactory cues can lead to memories that are stored in different neural substrates of the brain (Galizia et al., 2012; Giurfa and Sandoz, 2012; Menzel, 2012)

and guide the foraging bee toward the learned stimuli (Dukas, 2008).

Whenever a successful forager returns to the hive, it searches for nest mates to share the collected liquid food that is transferred via mouth-to-mouth trophallaxis. As foragers may carry the scent of the flowers diluted in the nectar inside its crop, sharing of scented food allows different worker groups, even those not directly involved in foraging-related activities like nurse bees, to learn the nectar scent and gain a key information about the currently exploited food source (Pankiw et al., 2004; Grüter et al., 2006). Operational cast in charge of unloading nectar (receivers) is mainly comprised of middle age workers. They can accept or refuse to unload nectar mainly based on its quality (gustatory cues) and the quality of the alternative sources that are currently exploited in the field (Seeley, 1989). If the incoming nectar is too diluted, receivers may refuse to unload it, a decision that affects food distribution through the nest together with the spread of olfactory and gustatory information (Ramírez et al., 2010).

According to the properties of the exploited floral patch and the food storing level of the colony, successful foragers can result stimulated to perform recruiting dances (von Frisch, 1967). This signal communicates among other aspects the location of profitable food sources encoded in the dancers' maneuvers (von Frisch, 1967; Dyer, 2002; Riley et al., 2005; Grüter and Farina, 2009). Several bees can simultaneously follow a dancer, including experienced foragers that can be reactivated to collect resources (Biesmejer and Seeley, 2005; Grüter et al., 2008) or novice foragers that search for reliable information to initiate their activities (von Frisch, 1967; Riley et al., 2005).

As we can see, honeybees display a rich and interesting behavioral repertoire, in which thresholds of response and associative learning play a fundamental role in the framework of foraging activities. Several protocols have been developed to address these two main plastic components that influence the honeybees' decisions. Taking advantages of the fact that honeybees extend their proboscises as a reflex response to antennal stimulation with a sufficiently concentrated sucrose solution (Kuwabara, 1957; Takeda, 1961; Bitterman et al., 1983), their response threshold to sugar can be approximate by the lowest concentration that elicits the extension of the proboscis within successive presentations of increasing sucrose solutions (Page et al., 1998; Pankiw and Page, 1999). Olfactory memories can also be quantified using the proboscis extension response (PER), as bees that have associated a conditioned odor with a nectar or pollen reward protrude the proboscis when that stimulus is delivered onto the antennae (Gerber et al., 1996; Sandoz et al., 2000).

Although many studies have focused on sensory and cognitive capabilities of foraging age honeybees, very little is known about the role and importance of early experiences in the development of these abilities, their impact on long-lasting foraging decisions and their eventual contribution to coordinate complex tasks at a social scale. Some recent reports addressed the question about how gustatory and olfactory information acquired early in the adult life modifies the honeybee underlying individual and social behavior as well as their concomitant neurobiological processes and substrates. In this review we center on these new evidences that focused on the honeybee foraging-related behavior.

CHANGES IN GUSTATORY RESPONSIVENESS IN YOUNG HONEYBEES AFTER THE INCOMING OF SCENTED FOOD

Honeybees assess the value of a nectar source according to their own perception (Scheiner et al., 1999, 2001) deciding, in turn, whether to forage on it or not. We know now that gustatory responsiveness rests on genetic bases (Page et al., 1998); however it can also be modulated by the environment (Pankiw et al., 2004; Martinez and Farina, 2008). Under controlled conditions, honeybees offered to forage high-concentration of a sucrose solution for 24 h presented higher thresholds to sucrose than those fed with low concentrated solutions (Pankiw et al., 2001, 2004), suggesting that previous foraging experiences can modulate the thresholds of this behavioral response in the short-term. Not only foragers, but pre-foraging bees are also able to adjust their sucrose response thresholds to sucrose. Qualitative changes in incoming nectar affect the behavior of 3–6 day-old members of the colony (Pankiw et al., 2004). Moreover, recent evidence indicates that receivers (pre-foraging bees of about 14 days of age) modify their sucrose response thresholds according to the quality of the food previously passed by the returning foragers (Martinez and Farina, 2008).

More experiments have been done to understand the role the young pre-foragers play in the propagation of gustatory and olfactory information of the food within the honeybee colony. Workers of the same age maintained under controlled conditions in the lab were subjected to different reward programs that changed in food quality. This experiment showed that 14-day-old bees have a higher modulation to adjust their response than bees of 7 days. Moreover, this study suggests that gustatory responsiveness of pre-foraging workers varied with the presence of a scent in the food. Interesting, workers that showed PER toward the odor that had been previously offered in their food (conditioned response, CR) presented higher responsiveness than those bees that did not extent their proboscises (Ramírez et al., 2010). Presence of stable memories and changes in gustatory responsiveness (measured as Gustatory Response Scores, i.e., GRSs) in middle age bees motivates the idea that olfactory memories could affect the sensitization to sugar (Ramírez et al., 2010). In the laboratory it has been described that immediately after a single association between the odor (as conditioned stimulus) and the sugar reward (as unconditioned stimulus) the learning process is dominated by a sensitization component (Menzel, 1999). Then, during paring the stimuli, sensitization to sugar could influence gustatory responsiveness by lowering the response thresholds of the conditioned bees.

In a second experiment performed in queen right colonies, Ramírez et al. (2010) showed that 6/9- and 12/16-day-old bees were able to increase their responsiveness 8 h after a controlled influx of scented food (**Figure 1A**). Concomitantly with changes in GRSs, olfactory memories to the incoming odor were clearly detected in 12/16-day-old bees over the 24 h period, but not in younger bees (**Figure 1B**). Interestingly gustatory responsiveness did not change in foragers (**Figure 1A**), indicating that not all the age groups respond equally to variations in chemosensory information. One possibility is that variations among bees of different ages were related to the currently activity the workers perform. Higher sensitivity to sugar in

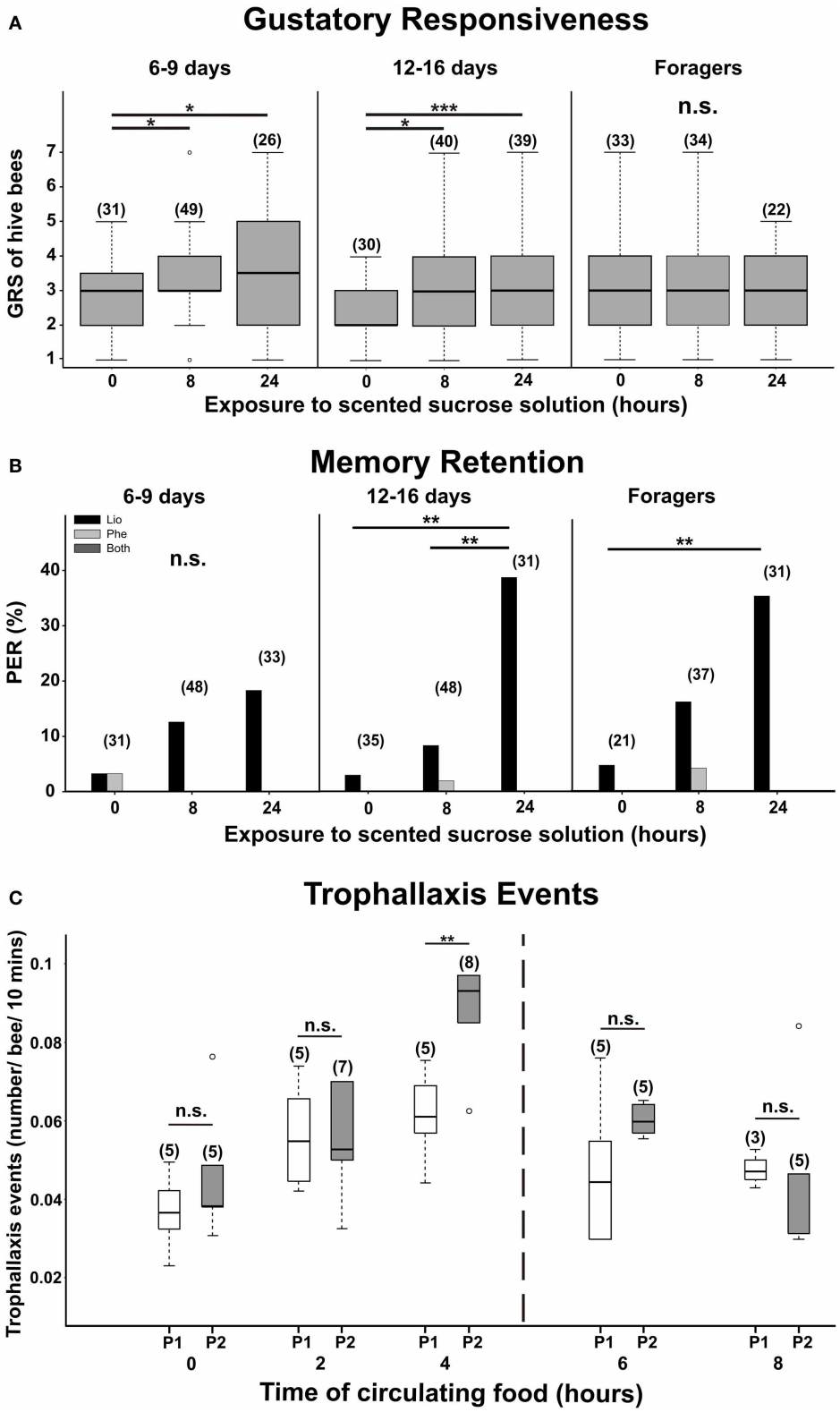

FIGURE 1 | Gustatory responsiveness and memory retention of free-flying bees after the influx of scented sucrose solution. Bees of different age groups: 6/9 days old, 12/16 days old, and foragers were captured from the hive. Gustatory response scores (GRSs) **(A)** and proboscis extension responses (PER) **(B)** to the odor solution (LIO, black bars), a novel test odor (phenylacetaldehyde, PHE, gray bars), or both (dark gray bars) were measured during the incoming of unscented sucrose solution (15% w/w)

(Continued)

FIGURE 1 | Continued
(0 h) or after 8 h and 24 h of foraging on a scented sucrose solution (linalool, LIO, 15% w/w). In addition, number of trophallaxis events per bee during a 10min-observation period was counted from the experimental colony while foragers collected 15% w/w sucrose solution for 8 h **(C)**. White boxes represent the reward program number 1 (P1) in which the colony collected unscented 15% w/w sucrose solution. Gray boxes represent the reward program number 2 (P2) in which bees fed for 4 h from a LIO-scented sucrose solution (15% w/w) and afterwards from an unscented solution of equal concentration. The asterisks indicate statistical differences (Panel **A**: $*p < 0.05$, $***p < 0.001$, n.s., not significant; Dunn comparison after Kruskal–Wallis test; Panel **B**: $**p < 0.01$; *G*-test; Panel **C**: $**p < 0.01$; Mann–Whitney test. After Ramírez et al., 2010. With permission).

middle age receivers might play a role during nectar distribution, adjusting the probability of accepting food from incoming foragers according to both gustatory and olfactory nectar characteristics (Pankiw et al., 2004). Lowering sucrose response threshold after memory formation could be thereafter a mechanism that, by increasing the occurrence of mouth-to-mouth food exchanges (trophallaxis) between incoming foragers and food-receiving bees, contributes to the coordination of collective tasks soon after an influx of scented nectar. Higher number of trophallaxis events quantified 4 h after a controlled influx of scented food into the hive (**Figure 1C**) supports this hypothesis.

CHANGES IN MEMORY RETENTION ACCORDING TO THE AGE: THE ROLE OF EARLY OLFACTORY EXPERIENCES AND THEIR CONSEQUENCES ON LATER MEMORY FORMATION

Prior studies described young honeybees as poor learners because they did not perform consistently under laboratory conditions until they were 6/7 days of age (Ray and Ferneyhough, 1997; Morgan et al., 1998; Ichikawa and Sasaki, 2003). Recent reports however, have shown that the honeybee behavior is more plastic at early ages than first thought (Arenas and Farina, 2008; Behrends and Scheiner, 2009). It is assumed that the poor learning performance in newly emerged honeybees is related to the ongoing development of the antennal lobe (AL), which is an important neuropile for olfactory information processing of the bee brain learning (Masson and Arnold, 1984; Morgan et al., 1998). Although part of the honeybee central nervous system involved in olfaction is fully innervated at 2 days adult emergence (Masson et al., 1993), it is believed that the formation of neural circuits concerned in olfactory learning is activity-dependent and young bees need to be subjected to a range of chemosensory stimuli to achieve good learning and memory abilities (Winnington et al., 1996; Farris et al., 2001; Maleszka and Helliwell, 2001; Ichikawa and Sasaki, 2003). Sigg et al. (1997) showed that volumetric increases of the AL glomeruli temporally correlate with activity-dependent improvement in learning performance. In this sense, Brown et al. (2004) showed that changes in the AL are dependent on the performance of foraging activities as the induction of precocious foraging behavior leads to significant increases in both the volume and the number of synapses in this olfactory processing center. Active-dependent maturation is further supported by responses measured in peripheral nervous system. Electrophysiological responses of olfactory antennal receptors increase steadily since emergence up to 4 days of age and remain high until 8 days of adult life (Masson and Arnold, 1987), point where the response to odors decreases if the bees are olfactory deprived (Masson and Arnold, 1984).

More evidence highlighting the plasticity at pre-foraging ages comes from experiments that measured the effect of early olfactory learning later in life (Arenas and Farina, 2008). These experiments showed that associative odor memories established as early as a few days after emergence can be retrieved when bees achieve foraging ages (17 days old; Arenas and Farina, 2008). Furthermore, the same study showed that retention of odor memories is not time-dependent and the learning events that occurred between 5–8 days of adult bees resulted in better olfactory retention than the same learning events occurring before (1–4 days old) or even after (9–12 days old) this period. Such an age-dependent effect of early learning could be observed in bees reared under laboratory conditions, where an odor diluted in the food for 4 consecutive days was the only "floral" odorant source the bees perceived in their whole lives. On the contrary, differences between age groups could not be seen in individuals reared inside the hive (i.e., memories established at both 5–8 and 9–12 days of age were equally well retrieved). Patterns of memory retention depending on the timing the experience took place and the rearing conditions (incubator vs. hive) emphasize the complex interplay between the age of acquisition and the environment during the development of the olfactory pathway.

Because consolidation of olfactory memories established at 5–8 days of age might take place through changes that modify structure-function relations when the olfactory system finally matures (Masson and Arnold, 1987; Masson et al., 1993; Winnington et al., 1996; Farris et al., 2001), early olfactory experiences could be important for the complete maturation of the neural pathways. To test whether olfactory memories established later in life are better retrieved if the honeybees have been previously exposed to an early olfactory stimulation, memories established in 5–8 or 9/12-day-old bees were tested after the exposure to a rewarded or unrewarded experience (Arenas et al., 2009a). Briefly, memories established at 5–8 or 9–12 days of age (by means of the offering of scented food, for details see Arenas and Farina, 2008; **Figure 2A**) were contrasted against those obtained in bees that, in addition to the latter experience, had been subjected to the offering of a second and different scented food at 1–4 or 5–8 days of age (**Figure 2B**), or exposed to a pure volatile compound delivered in the rearing environment (**Figure 2C**). Memories quantified in the PER-paradigm by the repeated presentation of the conditioned stimulus (CS) without reinforcement (i.e., 5-trials extinction test; Garelick and Storm, 2005) differed according to the timing and the nature of the prior sensory input (**Figure 2**). Memories recorded in bees pre-exposed to the rewarded olfactory input (**Figure 2B**) differed from those obtained in single-odor exposure bees (**Figure 2A**). These results indicate that early experiences either at the first 4 days of adulthood or at 5–8 days

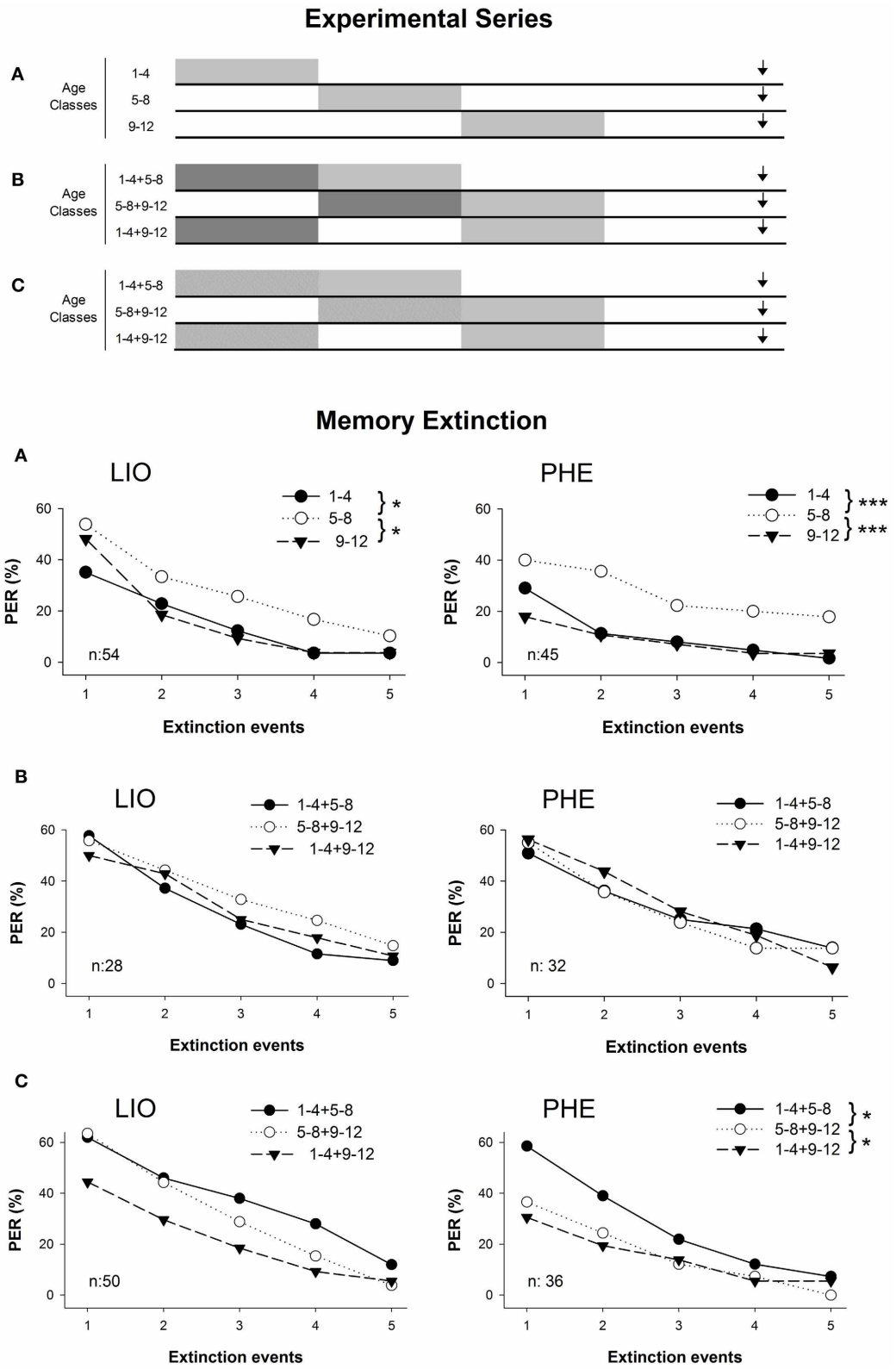

FIGURE 2 | Extinction response of early olfactory memories during five testing events in the proboscis extension response (PER). Schedules along the adult lifespan were indicated above for each experimental series. **(A)** Caged bees were offered a scented sugar solution for four consecutive days (gray boxes), and their olfactory memories evaluated at 17 days of age (black arrow). PER to LIO (left panel) or PHE (right panel) were tested when they were offered alone in the sugar solution. **(B)** In addition to the scented

(Continued)

FIGURE 2 | Continued
solution received for four consecutive days at 5–8 and 9–12 days of age (gray boxes, see **A**), an alternative scented food was previously offered (1–4 or 5–8 days old, dark gray boxes). As result three different treatments were obtained: 1–4 + 5–8, 1–4 + 9–12, and 5–8 + 9–12. **(C)** An odor was exposed as volatile compound for four consecutive days (crossed boxes) before caged bees were offered the scented sugar solution (gray boxes). Whenever LIO was used as the rewarded odor, PHE was used as the non-rewarded or exposed one and vice versa. The asterisks indicate statistical differences between age classes (***$p < 0.001$, *$p < 0.05$; *post-doc* comparison after RM-ANOVA test). The number of subjects within each experimental series was balanced and the number per treatment is indicated on each graph. (After Arenas et al., 2009a. With permission).

of age clearly enhanced the level of retention of odor-rewarded memories established later in life (at 5–8 or 9–12 days of age). Interestingly, memories established at 9–12 days of age were also improved by the pre-exposure of volatiles in the rearing environment, though its effect was weaker than the one found for the odor-rewarded experiences (**Figure 2C**). Results coming from rewarded and non-rewarded experiences that precede associative learning showed that relatively brief olfactory stimulations at the early stages of the adult bee's lifespan improve the memorizing process of new learning events.

Together, evidence suggests that young worker bees need to be subjected to the input of chemosensory stimuli, like odors in the food or in the rearing environment, to achieve proper associative learning and memory retention at older ages. Within the hive, bees are constantly exposed to diverse scents and young bees may have the chance to learn odors whilst performing tasks such as nursing or food processing. Thus, learning processes along the in-hive period might prepare workers for later tasks, including those such as foraging, which require the integration of complex cognitive abilities.

MORPHOLOGICAL AND FUNCTIONAL PLASTICITY OF FORAGING AGE BEES WITH EARLY ODOR-REWARDED EXPERIENCES

The AL (**Figure 3**) is the primary integrative center of odor information in the insect olfactory system. It is constituted of spherical subunits, the glomeruli, where olfactory receptor neurons from the antennae synapse with local interneurons and second-order neurons connecting with multimodal processing centers such as the mushroom bodies. Odors sensed by olfactory receptors are coded in the AL by patterns of glomerular activity (Friedrich and Korsching, 1997; Joerges et al., 1997; Galizia et al., 1998, 1999; Rubin and Katz, 1999; Sachse et al., 1999; Uchida et al., 2000; Carlsson et al., 2002; Sachse and Galizia, 2002). The arrangement and number of glomeruli that result activated by a particular odor is very well-conserved across adult honeybees; however, this neural code is dynamic and activity patterns can result modified by experience with odors (Faber et al., 1999; Sandoz et al., 2003; Rath et al., 2011).

During the last decade there has been a growing interest in using optical imaging techniques to explore odor-evoked neural activity in the ALs of insects (Joerges et al., 1997; Galizia et al., 1998, 1999; Sachse et al., 1999; Carlsson et al., 2002; Sachse and Galizia, 2002). Using this technique, Wang et al. (2005) recorded neural activity in honeybees of different ages. They showed that odor-evoked neural activity already occurs in the ALs of individuals as young as 1 or 2 days of age. Despite the relatively weak responses to odors in young bees, glomerular activity patterns were odor-specific, suggesting that the neural substrate for odor representation is already set up before emergence.

To study the changes induced by early associative learning events on the functional and structural properties of adult neural networks of the honeybee, the activity in the AL of 17-day-old honeybees which have experienced 1-Nonanol (1-NON) diluted in sucrose solution 5–8 days after emergence were recorded (Arenas et al., 2009b). This study showed that the conditioned odor evokes enhanced glomerular activity and modifies spatiotemporal response patterns. **Figure 3** shows how the glomerular maps of calcium responses evoked by 1-NON in control bees (naïve) and in bees subjected to early learning (T5–8) look like. Differences between the response patterns between these two groups are presented in **Figure 3B**. Map of relative response change shows the additional activation of glomeruli 23, 24, 36, and 62 as the main variation induced in learned bees (**Figures 3A–C**).

To investigate whether this reorganization translates into structural changes within the AL, the volume of 15 identified glomeruli in control and T5–8 bees, which had established memories with 1-Hexanol or 1-NON were also measured (Arenas et al., 2012). By comparing data from treated bees to bees without such experience, we showed that early olfactory learning results in the AL structural modifications (i.e., glomerular volume variations). Increases in glomerular volume appeared to be specific to the learned odor as 1-Hexanol and 1-NON long-term memories-induced changes in selective sets of glomeruli.

Comparison between volumetric measures and functional modifications in the AL network (i.e., calcium-imaging recordings) determined that those glomeruli showing structural changes after early learning were those that exhibited a significant increase in neural activity. Map of volume change in bees subjected to early learning events with 1-NON shows that the four newly recruited glomeruli (23, 24, 36, and 62) were those that also exhibited the largest volume increases (**Figure 3B**). Moreover, the hypothesis that glomerular volume changes took place in the same set of glomeruli that change the most in their odor-evoked activity was consistent with the glomerulus-wise correlation found between structural and functional changes (**Figure 3C**).

These results indicated that early olfactory learning results in long-lasting structural and functional modifications of the AL network in the form of glomerular volume variations and on the activation of new glomeruli upon olfactory stimulation with the odor that has been learned. It is then showed that early odor-rewarded experience induces a stable reorganization of olfactory circuits that accompanies the high plasticity of behavior, presumably because the olfactory system finishes its maturation at that stage (Masson and Arnold, 1987;

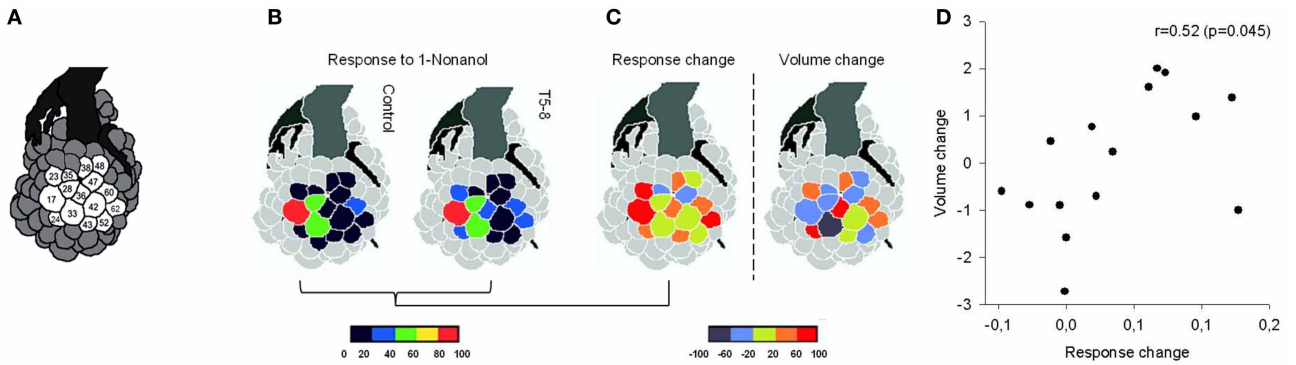

FIGURE 3 | Morphological and functional glomerular plasticity in 15 identified glomeruli of the honeybee antennal lobe (AL). (A) Schematic representation of the honeybee antennal lobe (AL) showing the 15 identified glomeruli of the AL labeled with their numbers. **(B)** Glomerular maps of calcium activity in response to 1-Nonanol in 17-day-old control bees (left) and in T5–8 bees (right), that were offered scented food 5–8 days after emergence. **(C)** Change in odor-evoked activity after early olfactory experience (left) and volume change obtained between control and experienced bees (right). Response intensity and volume changes were categorized in five equal bins from 0 to 100% or −100 to 100% respect (see Galizia et al., 1999; Hourcade et al., 2009). **(D)** Structural and functional changes were positively correlated after a glomerulus-wise Pearson correlation ($r = 0.52$, $P = 0.045$, df = 13). (After Arenas et al. 2012. With permission).

Winnington et al., 1996). This study demonstrated that the AL is a site in which both structural and functional plasticity can be observed following the formation of long-term olfactory memories (Grünbaum and Müller, 1998; Müller, 2000; Hourcade et al., 2009).

EARLY ODOR-REWARDED EXPERIENCES ON SOCIAL LIFE AND THEIR EFFECTS ON RECRUITMENT

Successful foragers perform waggle dances to communicate hive mates the location of the site they are visiting (von Frisch, 1967). In addition to spatial information, following bees can learn the odors of the exploited food source when small samples of food are shared via trophallaxis between the dancing bee and the follower during the short interruptions in-between dance maneuvers (Díaz et al., 2007). Floral scents learned in the recruiting context represent thereafter an important informational cue that assists recruited followers while search for the advertised goal (von Frisch, 1967; Dyer, 2002).

It has been shown that "unemployed" foragers that had visited a scented food source preferred to follow dancers carrying the odors they knew from previous field trips (Grüter et al., 2008). As a consequence, they were more likely to be reactivated to resume foraging tasks (Biesmejer and Seeley, 2005; Grüter et al., 2008). Biases in choice patterns however, may not be restricted to foragers that experienced the scent outside the hive, but may also involve followers that have experienced the floral scents inside the nest many days ago (Farina et al., 2005; Arenas et al., 2008; Grüter et al., 2009).

By introducing newly emerged color-marked bees into a glass-wall hive we study whether an influx of scented food offered at pre-foraging ages influence bees' interaction patterns and the chances of being recruited many days later (Balbuena et al., 2012). Eight days after introducing 70 ml of scented food into the hive by means of trained foragers, the number of color-marked bees either engaged in following dancers coming from distant feeders scented with the experienced odor or scented with a novel odor was quantified (**Figure 4A**). Bees that experienced the odor at 1–6, 4–11, and 8–13 days of age showed a stronger bias toward following the dancers carrying the experienced scent than with dancers carrying the novel scent (**Figure 4B**). Concomitantly with the increase in the number of bees following these dancers, more color-marked bees arriving at the feeders characterized with the experienced scent were observed (Balbuena et al., 2012; **Figure 4C**). Then, alteration in the patterns of interaction during dances suggests that previous experiences with the odor impact the chances of followers to be recruited by dancers that carried the experienced scent.

Preferences in the choice of dancing partners and biases in recruitment might be explained by stable and long-term olfactory memories acquired inside the hive during the influx of scented food 8 days ago (Arenas et al., 2007, 2008; Arenas and Farina, 2008; Grüter et al., 2009). The idea that dance followers acquired floral scent information while performing in-hive (non-foraging-related) tasks influenced recruitment on a long-term scale is consistent with the retrieval of olfactory memories established early in life and tested at foraging ages (Arenas and Farina, 2008; Grüter et al., 2009). Therefore, these results provide evidence that food-related information acquired by honeybees while performing in-hive tasks is functional (and putatively adaptive) in the recruiting context by facilitating the decoding of the spatial information transmitted in the waggle dance.

REMARKS AND CONCLUSIONS

A honeybee colony can forage several floral species simultaneously when available. Thus, each successful foraging bee brings back different types of nectar (differing in smell and taste) to the hive. Within this complex chemical environment, middle age workers involved in food reception can learn food odors through mouth-to-mouth trophallaxis while they unload and

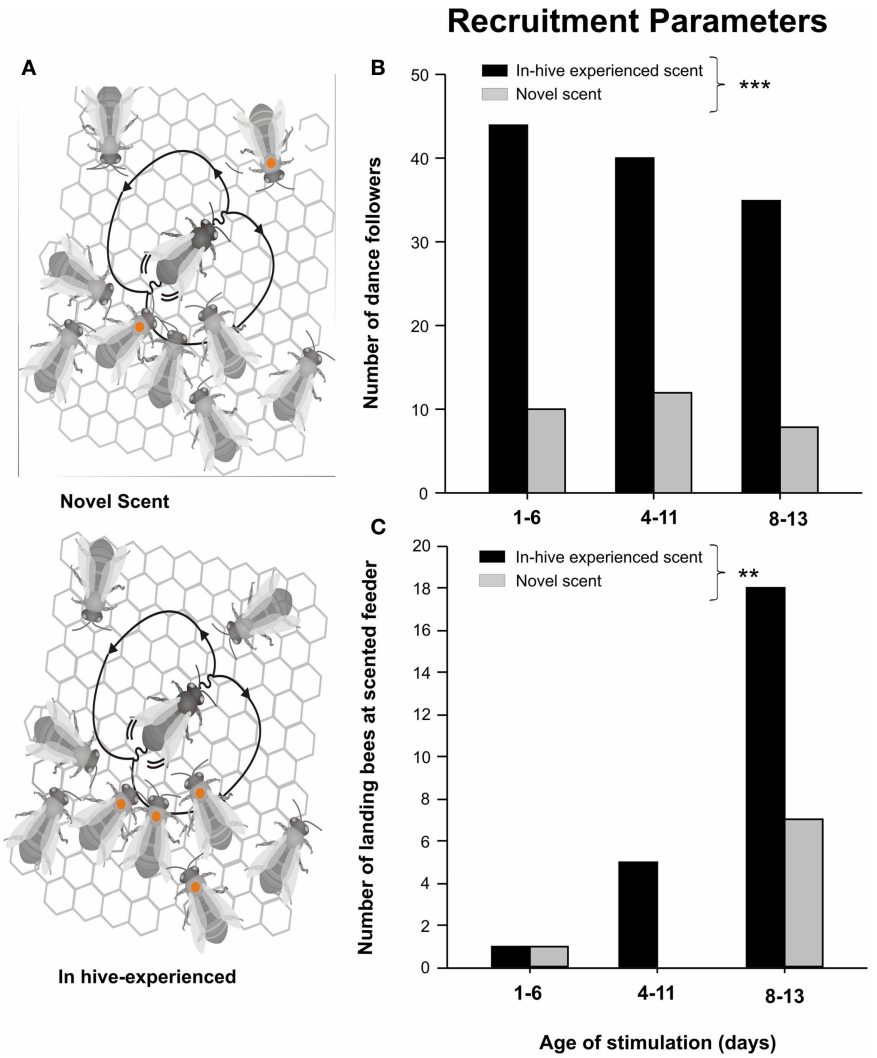

FIGURE 4 | Biased behavior within the recruiting context. (A) Schematic representation of the biased following behavior in both experimental situations: novel scent *(above)* and in-hive experienced scent *(below)*. **(B)** Number of dance followers engaged with dancers carrying either the experienced scent or a novel scent introduced 8 days ago by means of foragers. **(C)** Number of arrivals to the foraging location advertised by dancers carrying either the experienced scent or a novel scent. The asterisks indicate statistical differences between situations (number of dance followers: $^{***}p < 0.001$, after GLMM test; arrivals: $^{**}p < 0.01$, after Fisher's exact test). (After Balbuena et al., 2012. With permission).

store the scented food. One-week-old bees performing tasks such as nursing have similar opportunities when handle scented food directly from comb cells to feed the brood (Winston, 1987).

Although it was suggested that young honeybees did not learn consistently under laboratory conditions until they were 6/7 days of age (Ray and Ferneyhough, 1997; Morgan et al., 1998; Ichikawa and Sasaki, 2003), today there is enough evidences supporting that olfactory experiences gained soon after emergence are important to achieve proper learning and the abilities to form memories (Arenas and Farina, 2008; Arenas et al., 2009a; Grüter et al., 2009). Tuning the olfactory system by means of different rewarded and unrewarded odor inputs might prepare workers to face more complex tasks later in life. Consolidation of early odor memories (i.e., established at 5–8 days of age) may take place through changes that modify the structure and the function of the AL (first neuropile that process odor information), by the time the nervous system involving in olfaction goes through its final steps of maturation (Masson and Arnold, 1987; Winnington et al., 1996).

At the individual and social level the presence of long-lasting odor information might have important consequences during the resource exploitation. The novice foragers might be prompted to search for sources whose scents are reminiscent of the odors learned at early ages. Furthermore, retrieval of early olfactory memories may participate in the coordination of collective tasks, leading to non-random interactions between foragers and experienced followers within the dancing context. Biases in the choice of

dancing partners favor recruitment of foragers that despite naïve for the advertised food source, known in advance how it smells.

Floral odorant cues also alter how early and middle age bees perceive and respond to nectars of different qualities. Given the fact that the speed and extent the information propagates amongst nest mates rest on the quality and odorant cues of the food (Ramírez et al., 2010; Farina et al., 2012), individuals with a high plasticity in their response to changes in nectar characteristic have profound effects on the overall balance between foraging and processing capacity of the colony. In changing environments, accurate modulation of sensory-response systems in bees in charge of linking out and in-hive duties seems to be important to ensure the incoming of the best quality nectars available in the surrounding. To what extent variations in chemosensory information are triggered by associative learning in young adult bees remains a subject to study.

Abundance and composition of flower species have profound ecological consequences on pollinators since both can change over their short lifespan (Kearns and Inouye, 1993; Willmer and Stone, 2004). As long as rewarding floral types remain stable for a few weeks, early experienced bee would enhance the chances of the colony of foraging accurately and efficiently on these alternatives. Once the floral market changes and the odors precociously learned are no longer available by the time the bees initiate their foraging-related activities, the extraordinary neural plasticity of bees (Menzel, 1999, 2012; Giurfa, 2007) would allow adjusting their foraging preferences according to new and more profitable opportunities.

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