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Targeting a dual detector of skin and CO_2 to modify mosquito host seeking

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SUMMARY

Female mosquitoes that transmit deadly diseases locate human hosts by detecting exhaled CO_2 and skin odor. The identities of olfactory neurons and receptors required for attraction to skin odor remain a mystery. Here we show that the CO_2-sensitive olfactory neuron is also a sensitive detector of human skin odorants in both *Aedes aegypti* and *Anopheles gambiae*. We demonstrate that activity of this neuron is important for attraction to skin odor, establishing it as a key target for intervention. We screen ~0.5 million compounds *in silico* and identify several CO_2-receptor ligands, including an antagonist that reduces attraction to skin and an agonist that lures mosquitoes to traps as effectively as CO_2. Analysis of the CO_2 receptor ligand space provides a foundation for understanding mosquito host-seeking behavior and identifies odors that are potentially safe, pleasant, and affordable for use in a new generation of mosquito control strategies worldwide.

INTRODUCTION

Mosquitoes transmit deadly pathogens like malaria parasites, dengue viruses, and filarial worms to hundreds of millions of people every year. Female mosquitoes use two volatile cues to select and navigate toward hosts: exhaled CO_2 and human skin odorants (Cardé and Gibson, 2010; Dekker and Cardé, 2011; Dekker et al., 2005; Gillies, 1980; Mboera et al., 2000). Host preference and host seeking ability play pivotal roles in disease transmission and are targets for intervention.

Female mosquitoes detect plumes of exhaled CO_2 using a class of olfactory receptor neurons (ORNs) designated cpA. CpA neurons are housed in capitate peg (cp) sensilla on the maxillary palps and express the CO_2 receptor, comprising three conserved members of the *Gustatory receptor* (*Gr*) gene family (designated *Gr1*, *Gr2*, and *Gr3* in most mosquitoes, or *Gr22*, *Gr23*, and *Gr24* in *A. gambiae*) (Figure 1A) (Grant and O'Connell, 1996; Jones et al., 2007; Lu et al., 2007; Robertson and Kent, 2009; Syed and Leal, 2007). A host-seeking female will fly upwind when these neurons are activated, toward a CO_2 source in a laboratory arena or to CO_2-baited traps in the field (Cooperband and Cardé, 2006; Dekker et

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al., 2005; Healy and Copland, 1995; Lacey and Cardé, 2011; Xue et al., 2008). Conversely, preventing cpA from detecting changes in CO_2 dramatically reduces attraction toward CO_2 sources (Erdelyan et al., 2012; Turner et al., 2011).

The role of human odor in host seeking is more complex since it is a blend of hundreds of volatiles from skin, sweat, and associated microbiota (Bernier et al., 2000; Dormont et al., 2013; Gallagher et al., 2008) (see Table S1 for more references). ORNs in the antennae and palps express members of the *Or* and *IR* chemoreceptor families (Kwon et al., 2006; Lu et al., 2007; Pitts et al., 2011; Qiu et al., 2006; Syed and Leal, 2007) that respond to some skin odorants and are candidates for contributing to skin attraction (Carey et al., 2010; Wang et al., 2010). Other studies on antennal or maxillary palp sensilla have also identified activating odorants from skin (Ghaninia et al., 2008; Qiu et al., 2006; Syed and Leal, 2007). However, a causal relationship between activity of particular receptors or neuron classes and behavioral attraction has not been established as with the cpA neuron and CO_2. Of the odorants that have been tested, a small number, such as lactic acid, ammonia, carboxylic acids, 1-octen-3-ol, and nonanal, increase mosquito attraction when presented together with CO_2, but these are poor attractants by themselves (Njiru et al., 2006; Qiu et al., 2007; Syed and Leal, 2009), reviewed in Smallegange and Takken (2010). Mosquitoes are nonetheless attracted to whole skin odor even in the absence of CO_2 (Geier et al., 1999; Lacey and Cardé, 2011; Njiru et al., 2006; Schreck et al., 1981; Smallegange et al., 2010a). Intriguingly, mosquitoes that lack the co-receptor *orco*, and so lack functional *Or* receptors, are still attracted strongly to human skin odor with CO_2 (DeGennaro et al., 2013) suggesting that other receptors may also play a role in skin attraction.

Here we show that the CO_2-sensitive, *Gr*–expressing cpA olfactory neurons on the maxillary palps of mosquitoes are also sensitive detectors of human skin odor, a function conserved in *A. aegypti* and *A. gambiae*. We use a novel chemical strategy to selectively knock down cpA responses to skin odor and demonstrate that this neuronal pathway is also important for attraction to skin odor in a wind tunnel. The role of this neuron class in host-seeking behavior toward both CO_2 and skin odor establishes it as the key target for behavioral intervention. We screen ~0.5 million compounds *in silico* to identify new receptor ligands that modify mosquito behavior, including a cpA antagonist that reduces attraction to skin and an agonist that lures mosquitoes as effectively as CO_2. We demonstrate in *Drosophila melanogaster* that neuronal response and aversive behavior to a structurally diverse panel of odorants depends on the highly conserved CO_2 receptor. Our analysis of the CO_2 neuron ligand space provides a foundation for understanding mosquito host-seeking behavior and the chemical basis of host attractiveness and identifies odors that are safe, pleasant, and affordable for immediate use in mosquito control.

RESULTS

The cpA neuron plays a major role in attraction to human skin odor

As reported previously (DeGennaro et al., 2013), we find that *orco* mutant female *A. aegypti* mosquitoes without functional Ors retain strong attraction to a human skin odor source (Figure S1A,B), suggesting that other receptors may participate in attraction to skin odor. Since the CO_2 receptor neuron cpA is the only known ORN class in mosquitoes whose activity closely correlates with behavioral attraction, we hypothesized that volatiles from human skin may activate cpA. Indeed, human foot odor collected directly onto glass beads activates cpA in *A. aegypti* (Figure 1B). This corroborates a previously unexplained observation that cpA activity increases when a human hand is placed nearby (Kellogg, 1970).

To test whether cpA activation by human odor is important for attraction, we devised a novel chemical-based strategy to shut down the CO_2 receptor in *A. aegypti*. Butyryl chloride is a reactive volatile compound related to two of the strongest known inhibitors of the CO_2 receptor, butyraldehyde and butyric acid (Turner et al., 2011) (Figure 1C). A single puff of 1% butyryl chloride inhibits cpA from firing in response to subsequent CO_2 stimuli (not shown). We experimentally determined that a 3-min exposure to a small quantity (100μl, 10^{-2}) of butyryl chloride allowed to volatilize in an upended glass dish completely abolished cpA's subsequent responses to 1% CO_2 (Figure 1D,E) or exhaled breath (~4% CO_2; not shown) when tested ~5–20 min after exposure. CpA responses to butanone, a known ligand found in human skin odor (Turner et al., 2011), were also substantially reduced after pre-exposure (Figure 1D,E). Odor-evoked responses of the other two neurons in the same sensillum (cpB and cpC), which express members of the *Or* gene family, were not reduced by the treatment (Figure 1F). In fact, these neurons slightly increased in activity, as expected due to release of ephaptic inhibition between co-sensillar ORNs (Su et al., 2012). The inhibition of cpA is long-lasting and even after only a 60-s exposure requires between 12–24 hours to recover to control levels (Figure 1G).

Most importantly, the response of cpA to foot odor is completely lost after butyryl chloride exposure (Figure 1H). This effect is specific to the cpA neuron of the palp. The low response of the cpB and cpC neurons to foot odor is not affected by exposure (Figure 1I). Likewise, the summed response of antennal neurons to foot odor or to a synthetic blend of human odorants, measured by electroantennograms (EAG), did not change with butyryl chloride treatment (Figure 1J). Antennal responses to individual skin odorants were also unaffected by butyryl chloride pre-treatment (Figure S1C,D). While it is challenging to unambiguously identify specific antennal neuron classes, odorant responses of ORNs in individual sensilla can be compared before and after a puff of butyryl chloride. We find that odorant responses in many antennal trichoid sensilla are largely unchanged after butyryl chloride exposure (Figure S2).

The ability to specifically shut down cpA responses to human skin odor provides an ideal way to test whether the neuron is involved in attraction using a behavior assay where we can track many aspects of navigation. *A. aegypti* females will take off, navigate upwind, and land on a dish of foot odor–laden beads in a wind tunnel even in the absence of a CO_2 plume (Lacey and Cardé, 2011) (Figure 2). In 5-min assays, substantially fewer mosquitoes landed on the odor-laden beads after pre-exposure to butyryl chloride (Figure 2B). Analysis of flight videos showed that the proportion of pre-exposed, "cpA–off" mosquitoes that took off from the release cage was greatly reduced (Figure 2C, Movies S1–S4). Most sham-treated control mosquitoes took off relatively quickly toward foot odor, but the cpA–off mosquitoes that did take off did so with more delay (Figure S3). CpA–off mosquitoes that took off from the release cage showed no deficit in ability to fly (Figure S3, Movie S4). Of these, less than half successfully navigated to the beads; the behavior of others resembled that of no-odor controls (Figure 2D, Figure S3). Residual landing behavior observed in treated mosquitoes is likely mediated by short-range cues detected by ORNs other than cpA such as the *Or-* and *IR*-expressing neurons. In a separate control assay, pre-exposure to butyryl chloride did not impair mosquitoes' ability or preference for resting at the top of a small cage, or the increase in this preference when a warm, moist stimulus was introduced above the cage (Figure S4A,B), making general physical or behavioral deficits unlikely. Taken together, these results show that the highly conserved CO_2 receptor–containing neuron detects and participates in attraction toward human skin odor.

The CO_2 receptor neuron cpA is a sensitive detector of human skin odorants

In order to identify individual odorants from human skin that activate the cpA neuron, we first searched the literature for human-associated odorants other than CO_2 that interact with this neuron. We found two reports of such odorants: cyclohexanone (Lu et al., 2007); and butanone (Turner et al., 2011; Turner and Ray, 2009). Neither study explored possible connections with skin odor, but we used them and other known non-human ligands of the CO_2 neuron (Lu et al., 2007; Turner et al., 2011; Turner and Ray, 2009; data not shown), to manually select a panel of structurally related human-associated odorants for electrophysiology (Table S1). Over 35% of these odorants activated the neuron robustly (>30 spikes s^{-1}) in *A. aegypti* (Figure 3A). Although the anthropophilic *A. aegypti* and *A. gambiae* belong to divergent mosquito subfamilies, their CO_2 receptor genes are highly conserved (Robertson and Kent, 2009). Accordingly, we find that cpA responses to this panel of odorants are similar between these two species (Figure 3A), suggesting a conserved role in detecting host odor.

CpA's responses to these skin-derived odorants are dose-dependent (Figure 3B) and have similar temporal response profiles to CO_2 (Figure 3C). In its natural environment, a mosquito at a distance from a potential host will encounter plumes containing mixtures of CO_2 and skin odor at low concentrations. To test whether a mosquito would respond more sensitively to a combined stimulus, we measured cpA responses to binary mixtures of the two types of activators, CO_2 and skin odorants. We find that cpA's response to a mixture of CO_2 and skin odorant is additive, the response to the combined stimulus being significantly greater than its response to either stimulus alone (Figure 3D). This contrasts with *Or*-expressing neurons, where mixtures of two activating odorants do not elicit stronger responses than the stronger activator by itself (Münch et al., 2013). The additive effect is consistent with previous behavioral observations that anthropophilic mosquitoes are more attracted to a combination of skin odor and CO_2 than to either lure alone (Costantini et al., 1996; Dekker et al., 2005). Prior exposure to CO_2 or skin odorants, however, does not change neural responses to following stimuli (Figure S4C).

In silico identification of cpA activators and inhibitors desirable for human use

Since the CO_2 receptor (*Gr1*, *Gr2*, and *Gr3*)–expressing neuron cpA is critical for attraction to both exhaled CO_2 and skin odor, it is a high-priority target for manipulation of host-seeking behavior. In previous proof-of-principle experiments we identified cpA agonists, ultra-prolonged agonists, and antagonists (Turner et al., 2011; Turner and Ray, 2009), but these chemicals are unsuitable for use around humans due to unpleasant odors (rancid butter, sweaty, etc.) and health safety concerns. We sought to identify ligands that have stronger effects on cpA activity and are also pleasant smelling, safe, and affordable. We modified an *in silico* screen from a computational method we developed in *Drosophila,* which predicts new ORN ligands from a small set of known ligands (Boyle et al., 2013) (Figure 4A).We compiled existing data on odor-evoked activity for the conserved CO_2 receptor from *A. aegypti*, *A. gambiae*, *Culex quinquefasciatus*, and *D. melanogaster* to generate training sets for cheminformatic analysis (Lu et al., 2007; Turner et al., 2011; Turner and Ray, 2009; Figure 3A; unpublished data). Known ligands fell into multiple structural classes, suggesting the possibility of distinct binding pockets on the receptor. To improve the chances of identifying structural features for potentially distinct binding sites, we separated active compounds into three training sets: aromatic/cyclic ligands, straight-chain ligands, and ligands from both sets together.

We first identified a small subset of molecular descriptors whose values correlated highly with cpA activity among 3,224 molecular descriptors from Dragon (Talete) by Sequential Forward Selection (Boyle et al., 2013). We applied this process independently for each

training set, resulting in three separate activity-optimized molecular descriptor sets (Table S2). 3D and 2D molecular descriptors were preferentially selected, indicating that shape-related features were important for interaction with the receptor. We next ranked a library of >440,000 chemical structures (including ~3,200 volatiles from natural sources) by their computationally determined similarity to known ligands using the three optimized descriptor sets, generating three lists of predicted ligands that cumulatively represent >1000 potential ligands for the CO_2 receptor–expressing cpA neuron.

From these predicted CO_2 receptor ligands we judiciously selected 138 compounds for electrophysiological testing based on desirable characteristics for application such as smell, presence in natural sources, human safety profile, and cost to procure. Approximately 30% of the tested odorants activated the cpA neuron with >30 spikes s^{-1} (Figure 4B). To our satisfaction, ~85% of these activators are already approved for use as flavor, fragrance, or cosmetic agents, and many have been listed as "generally recognized as safe" (GRAS) by the Flavor and Extract Manufacturer's Association (Table S1). Several of these smell pleasant to humans, increasing their value for practical use in mosquito control.

Odorants in this screen were presented in a manner that raised cpA's background firing rate during stimulation by ~50 spikes s^{-1} (Figure S7B), thus also revealing a number of potential inhibitors (Figure 4B). 15 odors reduced the background firing rate. We retested these and all odorants that evoked <40 spikes s^{-1} in cpA in a secondary screen for ability to inhibit response to an overlaid 0.15% CO_2 stimulus (Figure 4C). Several compounds inhibited cpA to some degree; ethyl pyruvate strongly inhibited cpA (Figure 4C, Figure 5A, Table S1). A structurally related odorant, methyl pyruvate, also strongly inhibited cpA (Figure 4C, Figure S5A, Table S1). We found comparable inhibition at ~10 times lower concentrations than for previously reported inhibitors such as 1-hexanol (Turner et al., 2011).

Longer-term recordings with newly discovered activators also revealed an ultra-prolonged activator. After a 3-s exposure to (E)-2-methylbut-2-enal, cpA continues firing at ~45 spikes s^{-1} for at least 5.5 min (Figure 4D,E). CO_2 responses during this period are significantly reduced (Figure 4F), suggesting that this odor, which smells better (green fruit) than the previously reported ultra-prolonged activator butanedione (rancid butter), could disrupt navigation toward a CO_2 source (Turner et al., 2011). Taken together, the chemical informatics approach enabled us to rapidly identify compounds with greatly improved activity, safety, and smell for potential practical applications.

A cpA inhibitor reduces attraction of mosquitoes to skin

Since the cpA neuron detects skin odor and is important for attraction, an inhibitory odorant is predicted to block attraction of mosquitoes to skin. The cpA inhibitor ethyl pyruvate was selected for testing since it is listed as a GRAS compound, is approved as a flavor agent in food, and has a pleasant smell (fruity, sweet, rum, caramel) (Table S1). Ethyl pyruvate completely eliminates cpA responses to foot odor when they are presented together (Figure 5B). We modified an arm-in-cage repellency assay using gloves with chemical-treated mesh–covered windows to quantify attraction of *A. aegypti* mosquitoes to the human hand without exposing the hand to mosquito bites or direct contact with test chemicals (Kain et al., 2013). Ethyl pyruvate substantially reduced attraction, measured as the number of times mosquitoes landed on the mesh over a human hand (Figure 5C). Considered with the results of the previous wind tunnel experiments, we interpret that inhibition of the cpA neuron reduces attraction by masking detection of skin odor.

A cpA activator lures mosquitoes to a trap as effectively as CO_2

CO_2 is the primary lure in mosquito traps used for control and disease vector surveillance. Generating CO_2 involves burning fuel, evaporating dry ice, releasing compressed gas, or fermentation of sugar (Smallegange et al., 2010b) and is expensive, cumbersome, and impractical for use in developing countries where new control strategies are most needed. We tested whether an odorant that mimics CO_2-mediated activation of the cpA neuron can substitute for CO_2 as an effective lure. We generated dose response curves for two strong activators in both *A. aegypti* and *C. quinquefasciatus* (Figure 5D, Figure S5B). Cyclopentanone is a strong activator in both these species, is approved as a flavor and fragrance agent, is a GRAS substance, and has a pleasant minty smell (Table S1). CpA responds to repeated stimuli of cyclopentanone with a temporal activation profile that mimics its CO_2 response (Figure 5E,F) and tracks changes in levels of both compounds with similar temporal acuity (Figure 5E, Figure S5C), suggesting that mosquitoes will be able to navigate efficiently along plumes of this odorant. The strong and conserved cpA response, promising safety and fragrance profile, and ability to mimic CO_2 activation made cyclopentanone an excellent candidate for behavioral testing.

The efficacy of cyclopentanone as a lure was tested in semi-field experiments with *C. quinquefasciatus*, a mosquito present where the experiments were conducted in Southern California. We released 50 female mosquitoes overnight in a modified greenhouse with two counter-flow geometry mosquito traps, one baited with diluted cyclopentanone and the second with solvent (Figure 5G). Mosquitoes preferred cyclopentanone in a dose-dependent manner (Figure 5H,I). Remarkably, capture numbers for traps baited with the highest dose of cyclopentanone were comparable to those recorded for traps baited with CO_2 in similar trials performed in parallel (Figure 5H). The number of molecules of cyclopentanone released was ~176× less than the number of CO_2 molecules released to produce a comparable catch rate. To our knowledge, no other chemical has been able to successfully lure mosquitoes to traps in large numbers in the absence of CO_2, let alone at rates comparable with CO_2. For example, traps baited with 10% lactic acid, one of the few weak attractants of mosquitoes found in laboratory assays (Smallegange and Takken, 2010), did not catch significantly more mosquitoes than control traps (Figure 5I). Adding CO_2 to cyclopentanone at the highest catch rates did not further increase the trap catch over either odor alone ($p = 0.36$, $n = 5$–6, 50 mosquitoes per trial).

We used the same assay to test whether the cpA inhibitor ethyl pyruvate could mask detection of a CO_2 source. A CO_2-baited trap that also dispensed ethyl pyruvate caught significantly fewer mosquitoes than a control CO_2-baited trap (Figure 5I). These results suggest that compounds like ethyl pyruvate that block both CO_2 and skin odor attraction will be useful for development of spatial masking strategies against mosquitoes.

Odor space detected by the cpA neuron

Since cpA neurons sense critical host cues, we analyzed structural similarities and relationships of detected ligands in chemical space. The 3 sets of optimized descriptors (Figure 4A) include a total of 64 molecular descriptors representing structural features that predict cpA activity, so we used them to map the position of each tested skin odorant in 64-dimensional space, visualized in 3D chemical space by principle component analysis (PCA). Most active skin odorants are found in a small region of this chemical space (dark green dots, Figure 6A). Ligands predicted *in silico* and confirmed as activators by electrophysiology (light green dots, Figure 6A) populate regions that overlap with active skin odorants. Inhibitory odorants inhabit similar regions, suggesting that their effect may be mediated via similar binding sites on the CO_2 receptor (red dots, Figure 5A). Odorants that did not show activity are spread out in a non-overlapping region (gray dots, Figure 6A). 110

compounds previously tested on the *A. gambiae* odor receptor (*Or*) repertoire (Carey et al., 2010), while broadly dispersed, showed limited overlap with the cpA ligand space (black dots, Figure 6A).

Functional groups distribute widely in this chemical space (Figure 6B), and strong ligands include chemicals from diverse functional classes (Figure 6C). Presumably, aspects of 3D chemical structure bring these ligands closer together in chemical space than characteristics like functional group. We grouped ligands of the CO_2 receptor by structural similarity (Euclidean distance in 64D optimized descriptor space; Figure 6C). The resulting tree had roughly three branches, each populated by structurally distinct odor classes: substituted pyrazines and pyridines, other cyclic compounds, and short aliphatic chemicals. While an in-depth analysis of binding sites is not possible without structural information about the receptor, we note that while butyryl chloride pre-treatment abolishes cpA responses to CO_2 and dramatically reduces its responses to butanone, a short, aliphatic compound (Figure 1E), cpA responses to the cyclic compounds pyridine and cyclohexanone, while significantly reduced, are still substantial (Figure S5D), suggesting that these ligands may act via different binding sites on the receptor.

Increasing prediction accuracy through machine learning

Data from the newly tested odorants (Figures 3A, 4B,C) enabled us to further improve ligand predictions for the CO_2 receptor. Activities of all tested odorants were compiled and used to identify a single optimized descriptor set as before (Table S2). We next incorporated a machine learning approach called Support Vector Machine (SVM) (Cortes and Vapnik, 1995) into our prediction algorithm. A 5-fold cross-validation indicated that the SVM-based approach had a substantially higher area under curve (AUC) of the receiver operating characteristic (ROC), signifying improved ligand prediction (Figure 6E). We obtained and tested 19 compounds from the top 200 ligands predicted this way, of which 12 activated the cpA neuron >30 spikes s^{-1}, and 2 inhibited the neuron, yielding an improved success rate of 74% (Figure 6F, Table S1). Six of these new ligands are present in human skin emissions and may play a role in skin attraction.

Structurally diverse odorants are detected by the conserved CO_2 receptor

A question that emerged is whether cpA's responses to structurally diverse ligands are mediated by the heteromeric CO_2 receptor encoded by *Gr1*, *Gr2*, and *Gr3* or by other receptors from the *Or* or *IR* families that may be present in the same cell. In order to address this question, we turned to the genetically tractable *Drosophila melanogaster*, whose CO_2-sensitive ab1C neurons express *Gr21a* (ortholog of *Gr1*, paralog of *Gr2*) and *Gr63a* (ortholog of *Gr3*). We tested a small panel of cpA ligands in *orco* (*Or83b^2*) mutant flies (with no responses in *Or*-expressing neurons, leaving the ab1C neuron easy to distinguish) and found that these odorants also activate ab1C (Figure 7A,B). Furthermore, the cpA inhibitor ethyl pyruvate also effectively inhibited the ab1C neuron (Figure 7A). Thus these diverse chemicals are sensed in *Drosophila* by the *Gr21a/Gr63a*–expressing CO_2-sensitive neuron, independently of *orco*. Although the *Drosophila* neuron's responses to these odorants were of a lower magnitude than in mosquito cpA, they were sufficient to test whether they depend on the *Gr21a/Gr63a* heteromeric receptor. In flies that lacked *Gr63a*, all odor-evoked responses were eliminated (Figure 7C). Activation of the CO_2 receptor in *Drosophila* elicits robust avoidance in a T-maze (Suh et al., 2007; Suh et al., 2004). Consistent with our electrophysiology results, we find that cyclopentanone is a strong repellent in a *Gr63a*-dependent manner, just like CO_2 (Figure 7D).

The ability to knock down responses by butyryl chloride pre-exposure was also partially conserved in the *Drosophila* CO_2 receptor. We found significant reductions in neuronal

responses of treated flies to a range of ab1C activators including CO_2 (Figure S6A). Behavioral assays after butyryl chloride pre-exposure showed a concomitant reduction in behavior (Figure S6B).

The simplest interpretation of these results is that the *Gr*-encoded CO_2 receptor is required for detecting odorant ligands as well as CO_2, and that this is evolutionarily conserved. The three broad ligand classes and CO_2 appear structurally different, and in the future it will be interesting to test whether they bind to different regions of the heteromeric CO_2 receptor.

DISCUSSION

Here we show that the *Gr1*, *Gr2*, and *Gr3*–expressing cpA neurons on the maxillary palps of mosquitoes play a critical role in attraction toward humans by detecting both exhaled CO_2 and odorants from skin (Figure 7D). In the absence of available mutants, we developed a novel chemical-based strategy to shut down odor-evoked neural responses in cpA and demonstrate that attraction toward skin odor is substantially reduced. We find an essential role for cpA in host-seeking behavior, partly explaining how mosquitoes that lack functional Or odorant receptors are still attracted to sources of skin odor, even in the absence of CO_2. We identify individual odorants from skin that strongly activate the CO_2 neuron, providing a foundation for understanding how differences in abundance of these chemicals on skin may contribute to host selection.

While we demonstrate that the cpA neuron is an important component of attraction behavior in a wind tunnel, we anticipate that the large number of *Or* and *IR*–expressing neurons in the antenna and maxillary palp, many of which also detect components of human odor, may participate in more challenging situations such as host and landing site selection. It will be interesting to apply the chemical-based approach we have developed or genetic strategies to investigate the contribution of antennal *Or* and *IR* genes.

Our findings also suggest that cpA and its highly conserved *Gr* receptors are even higher-priority targets than previously believed for manipulating mosquito host-seeking behavior. We use an *in silico* approach to screen >440,000 odorant-like compounds followed by electrophysiological validation to rapidly identify strong cpA activators and inhibitors that are safe to handle, pleasant smelling, and affordable. The success of this method suggests that cheminformatics can be used for "computational reverse chemical ecology" to identify important odorant cues from a complex source by studying predicted ligands of a receptor.

From these ligands, we identify an ideal inhibitory odorant, ethyl pyruvate, which is approved for use as a flavoring agent and has a pleasant smell. We show that ethyl pyruvate blocks close-range attraction toward a human hand, consistent with other experiments showing a role of cpA neuron activity in attraction to skin. We also identify cyclopentanone as an ideal activator since it has a pleasant aroma and is regarded as a relatively safe chemical (Belsito et al., 2012). Traps baited with this activator catch a comparable number of mosquitoes as CO_2, even though they release far fewer molecules.

Our findings have wide-reaching implications for control of mosquito-borne disease. While DEET is effective as a short-range olfactory repellent, its use is extremely limited in disease-prone areas such as Africa and Asia. The recent discovery of insect DEET receptors and substitutes offer great promise (Kain et al., 2013), however a combinatorial approach provides far greater potential. The CO_2 receptor agonists and antagonists we identify can be used in two additional powerful and complementary approaches for next-generation mosquito control using "mask" and "pull," similar to push–pull strategies (Cook et al., 2007): inhibitory odorants will mask human scent, and activating odorant–baited traps will

pull mosquitoes away (Figure 7D). Several of the odorants we identify are GRAS, so the approval process for human use will be expedited and affordable. Moreover, our estimates suggest that the odorant lures like cyclopentanone can be affordable for use for surveillance and control traps in countries affected most by mosquito-borne disease. These approaches have the potential to protect large areas, may not need direct application on the skin, and present economical and environmentally friendly ways to disrupt host seeking of vector species like *A. gambiae*, *A. aegypti*, *C. quinquefasciatus*, and other disease-carrying insects around the world.

EXPERIMENTAL PROCEDURES

Details are available in Extended Experimental Procedures in Supplementary Materials

Electrophysiology

Adult female mosquitoes and *Drosophila* were tested 3–14 days after emergence with electroantennography (EAG) and single-sensillum extracellular recordings (SSR) as described previously (Bjostad, 1998; Turner et al., 2011; Turner and Ray, 2009) with modifications. CpA–off and sham treated mosquitoes were pre-treated for 1 min or 3 min in an upended 1L glass dish in which 100 μl of 1% butyryl chloride or paraffin oil was left for 10–20 min. Human odor was collected on glass beads worn in socks for ~6 hrs, and 20 ml beads were placed inside a 25 ml disposable pipette; a Syntech CS-55 was used to redirect an airstream from a comparable cartridge with clean beads to generate a controlled stimulus (Figure S7A). Control responses to clean beads were subtracted from the results reported; cpA neurons with >20 spikes s^{-1} response to a control puff were not considered.

All chemical odorants were dissolved at 10^{-2} in paraffin oil or water, with 50 μl per odor delivery cartridge except where indicated. For the experiment with binary mixtures of CO_2 and activating odorant (Figure 3D), the carrier airflow was adjusted to keep total airflow constant, allowing quantitative comparison between conditions. The odor delivery system was modified as shown in Figure S7B for screens in Figure 4B and Figure 6F; solvent responses during the same recording session were subtracted. For experiments with ultra-prolonged activators, a controlled 3-s stimulus of (E)-2-methylbut-2-enal (200 μl 10^{-1} in paraffin oil on filter paper) was delivered from a 10 ml disposable pipette into the carrier airstream. Subsequent CO_2 stimuli were delivered using a MNJ-D microinjector (Tritech Research). Activity was calculated by subtracting baseline activity 1 s prior to each stimulus. Spike counting was done manually or with Igor Pro 6.2 (Wavemetrics) with the Neuromatic v2.00 macro (Jason Rothman).

Electroantennograms were recorded from decapitated mosquito heads, with the neck tissue inserted into a saline-filled glass reference electrode and a recording electrode placed onto the cut tip of the antenna. EAG traces were normalized to the reference odor 3-methyl-1-butanol and analyzed in Clampfit 10.3 (Molecular Devices).

Behavior

Two-choice cage assay—Twenty-five to thirty 10–12-day-old female *A. aegypti* were starved overnight in a (30 cm)^3 cage with a glass top; trials were conducted between 1430 and 1900 hrs. A transparent partition separated the experimenter from the cage, which was left undisturbed 10 min before the start of each assay. Each cage was used no more than once hr^{-1}. An odor-laden sock (95% nylon/5% spandex shoe liner worn ~6 hrs) and a clean sock were hung from either side of the cage, side alternating between trials, and mosquito behavior video-recorded for 5 min. One trial with <30% participation was excluded from

analysis. Preference index = (# mosquitoes on "odor" side of test cage – # on "clean side) / (total # mosquitoes on either side), excluding resting mosquitoes that did not move.

Wind tunnel—Experiments were performed as described previously (Turner et al., 2011) with modifications. Room air (27°C; 35–40% relative humidity) was carbon filtered and drawn through a glass wind tunnel (36 cm × 40 cm × 128 cm) in a laminar flow at a constant rate of 0.2 m s^{-1}. Odor-laden or clean control beads as for electrophysiology experiments were elevated 7 cm above the floor of the wind tunnel in a covered 10 cm–diameter petri dish, 50 cm upwind from the release cage (Figure 2A). 8–14-day-old female *A. aegypti* were held in individual release cages without access to food or water for 17–23 hr at 27° C and ~70% relative humidity and pre-exposed to butyryl chloride or solvent as above immediately before testing. Each mosquito was kept in the release cage until still for at least 60 s (within 4 min of placement), then covers over the beads and the release cage exit were removed to start the assay, which was video-recorded for 5 min or until the mosquito landed or walked onto the beads. Trials were conducted between 1400 and 1830 h. Data from days with poor positive control responses were not considered.

Short-range attraction—Ten 6-day-old female *A. aegypti* were starved 30 hrs in a 7 cm diameter × ~5 cm high cage with wire mesh on one side. Test cages were placed inside an aquarium and left undisturbed 5 min, after which a filter paper soaked with 400 μl water and a beaker with 750 ml 40°C water were placed 5 mm above the cage (Figure S4A). Mosquito behavior was video-recorded for 3 min.

Arm-in-cage—Experiment was performed as described (Kain et al., 2013). A hand in a glove with solvent-treated mesh was placed in the test cage for 5 min, then the same hand in a test glove was replaced in the same cage for an additional 5 min; all trials were video-recorded for analysis. The outer mesh covering the glove window was treated with ethyl pyruvate (500μl, 10% in acetone) or solvent; solvent was allowed to evaporate before glove assembly.

Semi-field trapping—Two modified greenhouses at the Agricultural Experiment Station at the University of California, Riverside, were used as described (Turner et al., 2011) with modifications. Fifty laboratory-reared, mated, non–blood fed, female *C. quinquefasciatus* aged 8–14 days and starved for 24 hrs were released each evening around 1700 hrs and traps collected at ~0700 hrs. Each greenhouse contained counterflow geometry traps baited with CO_2 (250 ml min^{-1}, ~670 mmol hr^{-1}) or odorant.

T-maze—Experiments were performed as described (Turner and Ray, 2009) with modifications. For cyclopentanone trials, 10 μl of 1% odorant was allowed to volatilize ~2 min before testing. Preference index = (# flies in test arm – # flies in control arm) / (total # flies). Butyryl chloride pre-exposure was carried out as above on groups of flies housed in a 1 oz perforated, mesh-covered container during exposure (40–50 flies per container). Flies were tested <30 min after treatment.

Chemical informatics

Optimized descriptor sets—Descriptor optimization to CO_2-receptor ligands was done as described (Boyle et al., 2013) with modifications. Ligands that evoked >30 spikes s^{-1} were classified as activators, and those that reduced baseline firing rate by >5 spikes s^{-1} were classified as inhibitors. Descriptors were optimized independently for each of 3 training sets (aromatic/cyclic ligands, straight-chain ligands, and a combined set), resulting in 3 unique descriptor subsets (Table S2), and then combined into a single set of 64 descriptors representing molecular features that predict CO_2 receptor activity. Optimized

descriptor values were used to cluster active ligands, perform principle component analysis (PCA), and rank the library of >440.000 chemical structures for closeness to known ligands.

Ligand prediction using SVM—A receptor-optimized descriptor set (Table S2) was calculated based on ligand activity data for *A. aegypti* alone (this study; unpublished data). This descriptor set was used to train a Support Vector Machine (SVM) using regression and a radial basis function kernel available in the R package e1071 integrating libsvm (Chang and Lin, 2001; Karatzoglou et al., 2006); optimal gamma and cost values were determined using the Tune.SVM function. Twenty independent 5-fold cross-validations of the computational approach were done. The previously tested odors were randomly divided into 5 equal sized partitions and 4 of the partitions were applied to train the SVM. The remaining partition was used to test predictive ability. This process was repeated with each partition excluded and used to test predictive ability exactly once. A single receiver operating characteristic (ROC) curve for all 20 independent validations was plotted. The trained SVM was then applied to predict activity for a library of >440,000 compounds. Predicted ligands were screened for organoleptic odor profile using a flavor and fragrance database (www.thegoodscentscompany.com). Compounds that did not have foul smells and were categorized as flavor, fragrance or cosmetic agents were considered for purchase. A few additional compounds were also selected after cross-checking MSDSs and other literature to avoid toxins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

- Mosquito CO_2 neurons (cpA) detect human skin odor and are important for attraction

- *In-silico* screen of >440k chemicals finds excellent agonists and antagonists of cpA

- Blocking cpA activity abolishes attraction behavior toward human skin odor and CO_2

- An agonist lures mosquitoes to traps: a new generation of safe, affordable control
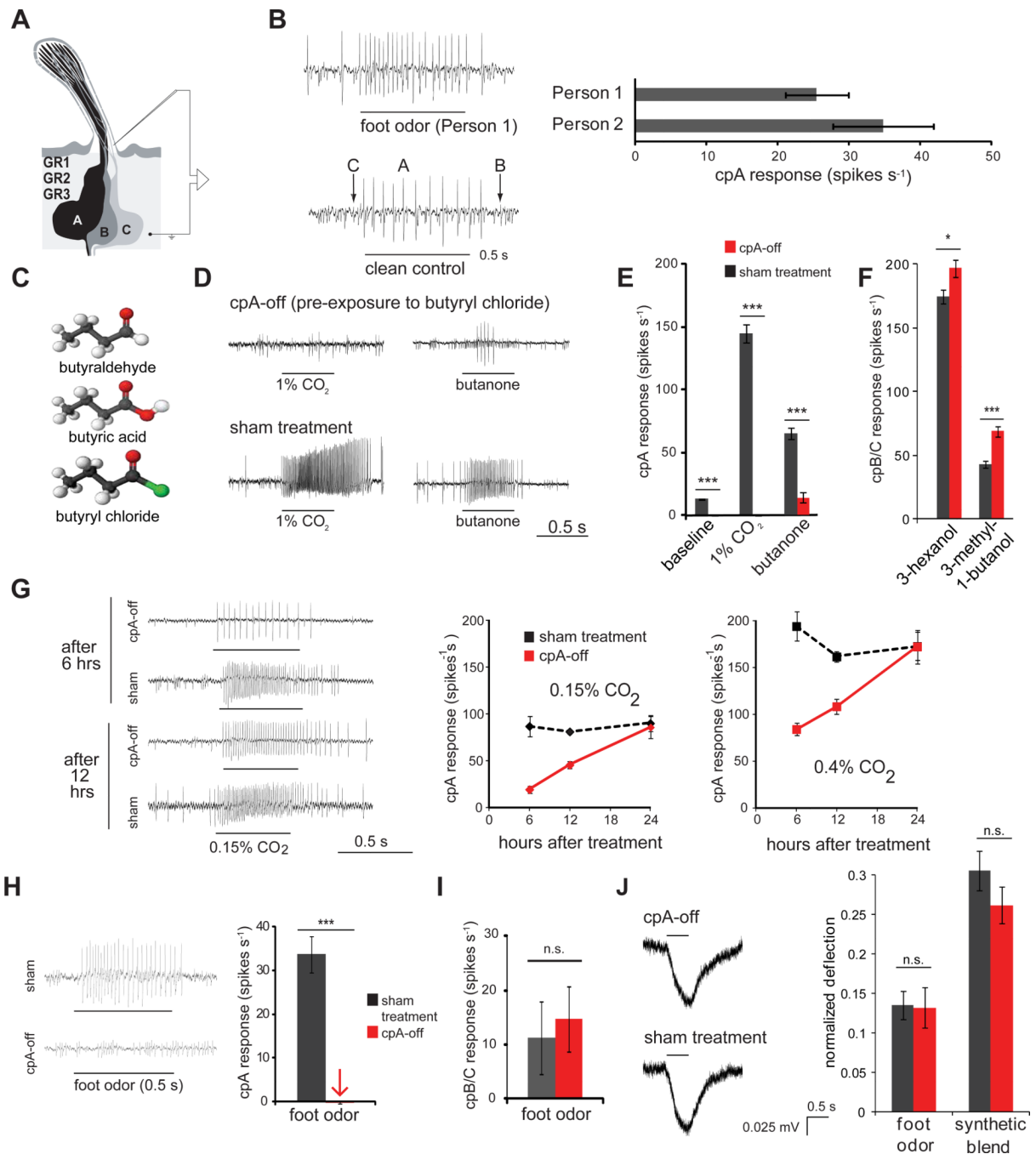
Figure 1. The CO$_2$-sensitive mosquito cpA neuron detects human skin odor
(A) Schematic of the maxillary palp capitate peg sensillum with three ORNs and electrodes for physiology. **(B)** Representative traces and mean change in firing rate of the *Aedes aegypti* cpA (large amplitude) neuron to foot odor carried on glass beads. $n = 6$–7. **(C)** Chemical structures of known cpA inhibitors and butyryl chloride. **(D)** Responses to indicated odorants after pre-exposure to butyryl chloride (10^{-2}) (cpA–off) or solvent (sham treatment). **(E)** Mean odor-evoked responses of the cpA neuron in cpA–off and sham treated mosquitoes and **(F)** combined odor-evoked responses of the two neighbouring neurons, cpB and cpC. **(E,F)** $n = 16$. **(G)** Sample traces and mean cpA responses to CO$_2$ after treatment.

(H) Sample traces and mean cpA responses to foot odor (mixed beads from Persons 1 and 2) after treatment. $n = 8$–9. **(I)** Summed cpB and cpC neuron response; $n = 4$–8. **(E,F,H,I)** Analyzed by ANOVA nested across 3–6 individuals. **(J)** Averaged traces and mean normalized electroantennogram (EAG) responses to foot odor and to a synthetic blend of human odorants. $n = 16$–18 (foot odor), 8–9 (synthetic blend); analyzed by t-test. * $p < 0.05$, *** $p < 0.001$. CpA–off mosquitoes were pre-exposed to chemical for 1 min **(G)** or 3 min (elsewhere). See also Figures S1, S2, and S4.
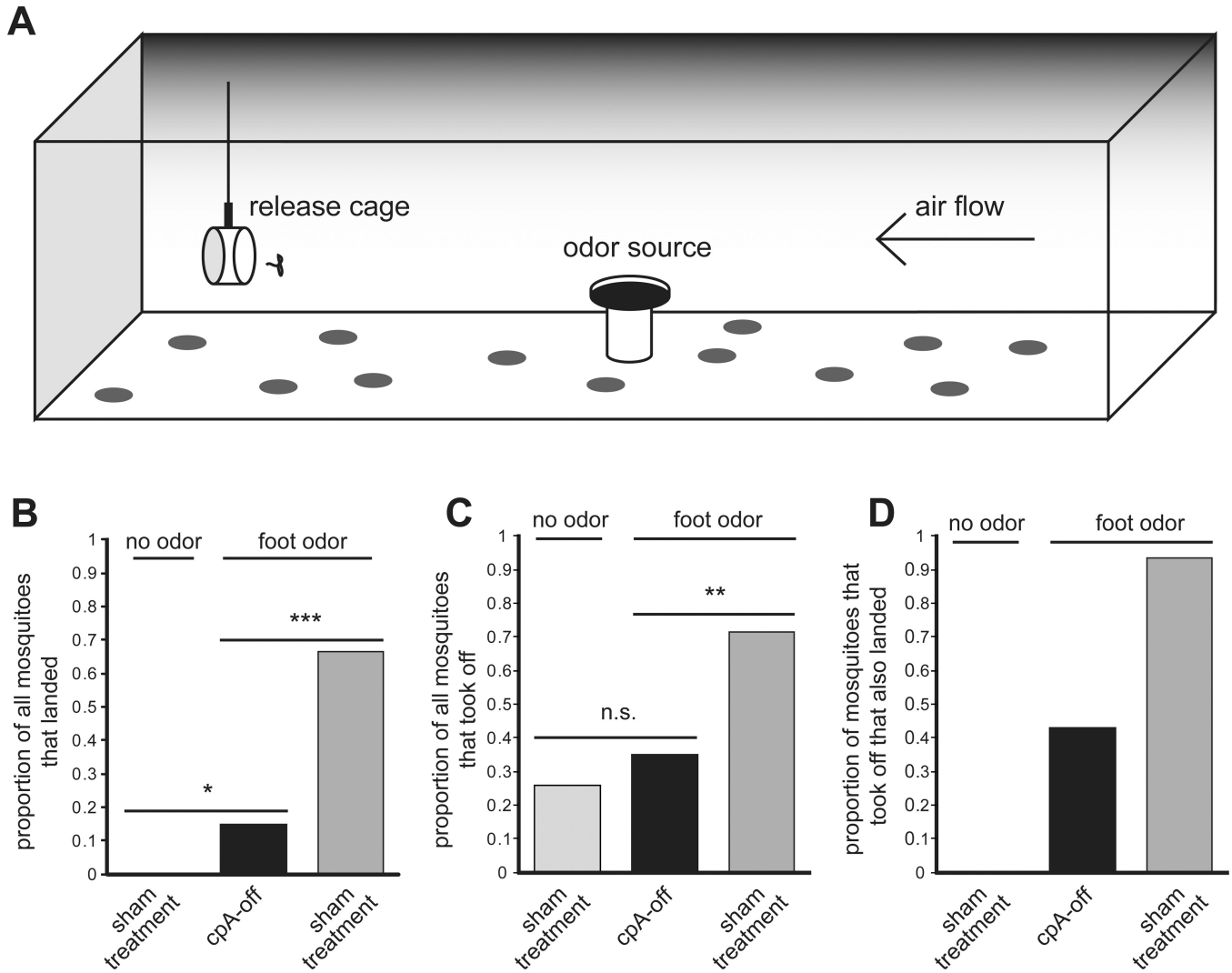
Figure 2. Activation of cpA is required for navigation of female *A. aegypti* toward a human skin odor source

(A) Schematic of wind tunnel assay for navigation of single female *A. aegypti* to glass beads worn in socks. Dark circles at the bottom provide visual cues for flight. (B) Proportion of butyryl chloride–exposed and sham-treated mosquitoes presented with beads with no odor or foot odor that landed on beads, or (C) took off from the release cage. (D) Proportion of mosquitoes that did take off that succeeded in landing on the beads. (B,C) $n = 20$–23 individuals per condition; analyzed by one-tailed proportion Z-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Error bars are s.e.m. See also Figures S2–S4 and Movies S1–S4.
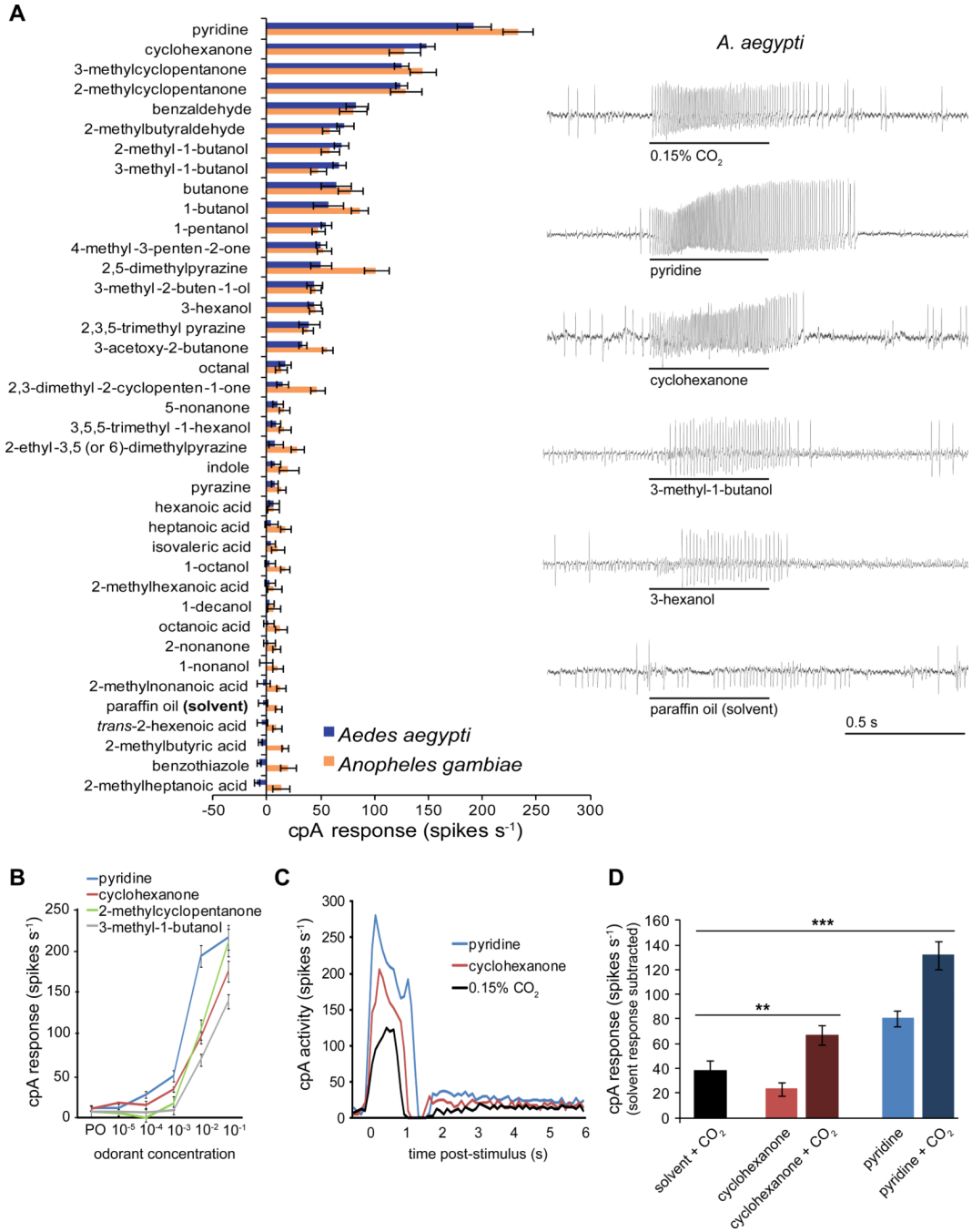
Figure 3. Specific odorants in human skin activate the cpA neuron
(A) Representative traces and mean responses of the cpA neuron to 0.5-s pulses of individual components of skin odor in *A. aegypti* and *A. gambiae*. $n = 4$–7. **(B)** Dose responses of *A. aegypti* cpA to representative activating odorants. **(C)** Mean firing frequency of *A. aegypti* cpA during a 1-s stimulus, counted in 100-ms bins. Responses averaged across $n = 4$–5 stimuli repeated in 15 s cycles. **(D)** CpA responses to combinations of skin odorants (at 10^{-3}) and 0.1% CO_2. Airflow was adjusted to maintain concentrations of each odorant. $n = 6$; one-tailed paired *t*-tests. ** $p < 0.01$, *** $p < 0.001$. Error bars are s.e.m. See also Figure S4 and Table S1.
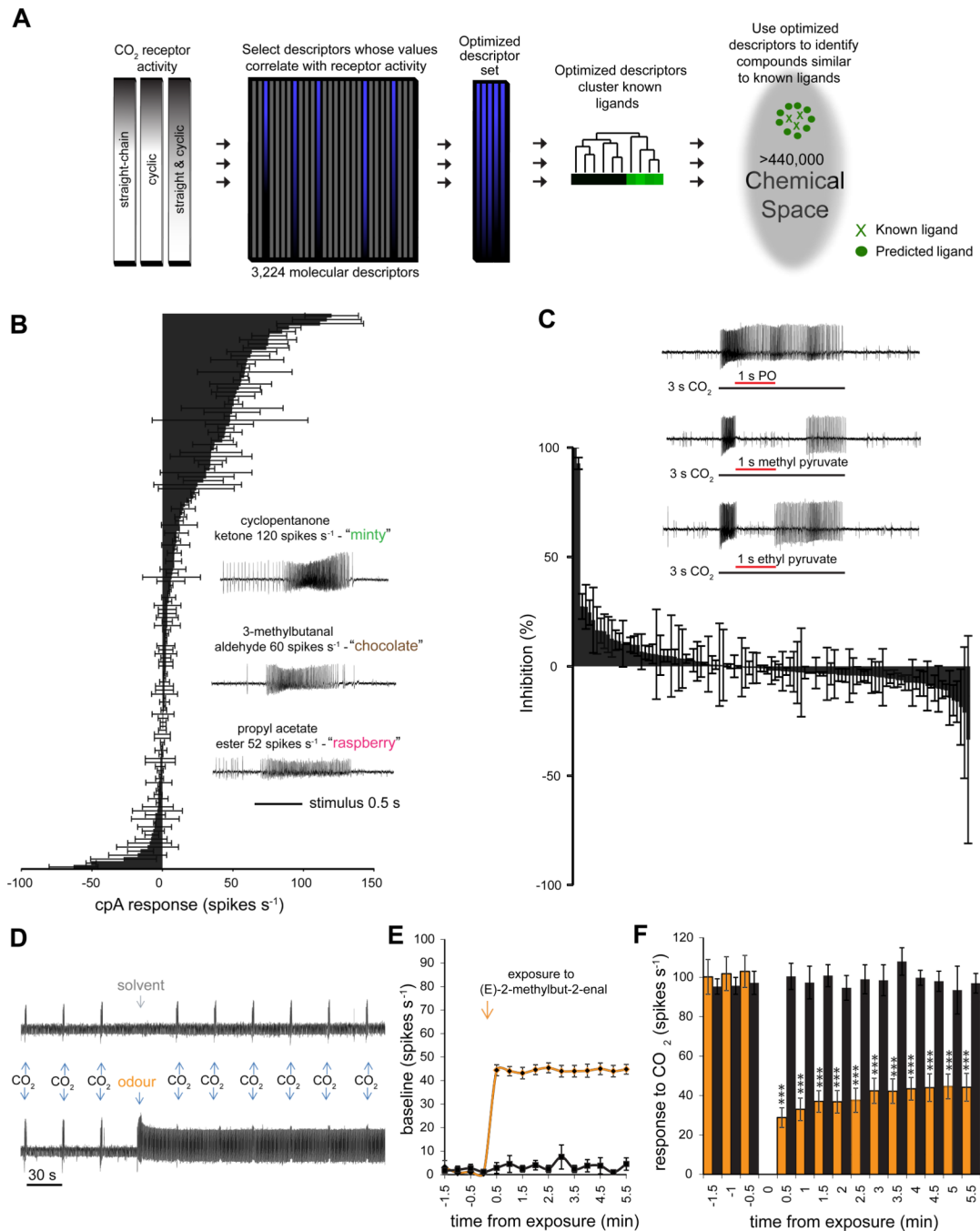
Figure 4. Identification of CO$_2$ receptor neuron activators, inhibitors, and ultra-prolonged activator in *A. aegypti*

(A) Overview of the cheminformatics pipeline used to identify novel cpA ligands from a large untested chemical space. **(B)** Representative traces and mean responses of the *A. aegypti* cpA neuron to 0.5-s pulses of 138 predicted compounds. Responses to solvent have been subtracted. $n = 2–6$. **(C)** Representative traces and mean percent inhibition (compared to solvent) of cpA by a panel of 107 odorants presented as a 1-s stimulus overlaid on a 3-s 0.15% CO$_2$ stimulus. $n = 2$, except for ethyl and methyl pyruvate, $n = 6$. **(B,C)** Error bars are s.d. **(D)** Representative traces from the cp sensillum to 1-s pulses of 0.15% CO$_2$ prior to and following a 3-s exposure to either solvent (paraffin oil) or (E)-2-methylbut-2-enal (10^{-1}).

(E) CpA baseline activity in the 1 s prior to each stimulus after exposure to odorant. **(F)** Mean responses of the cpA neuron to 1-s pulses of 0.15% CO_2, calculated by subtracting 1 s of baseline activity prior to each stimulus after exposure to paraffin oil (gray) or (E)-2-methylbut-2-enal (10^{-1}) (orange). n = 5–6 individuals; t-test, *** $p < 0.001$. **(E,F)** Error bars are s.e.m. See also Figure S5 and Tables S1–S2.
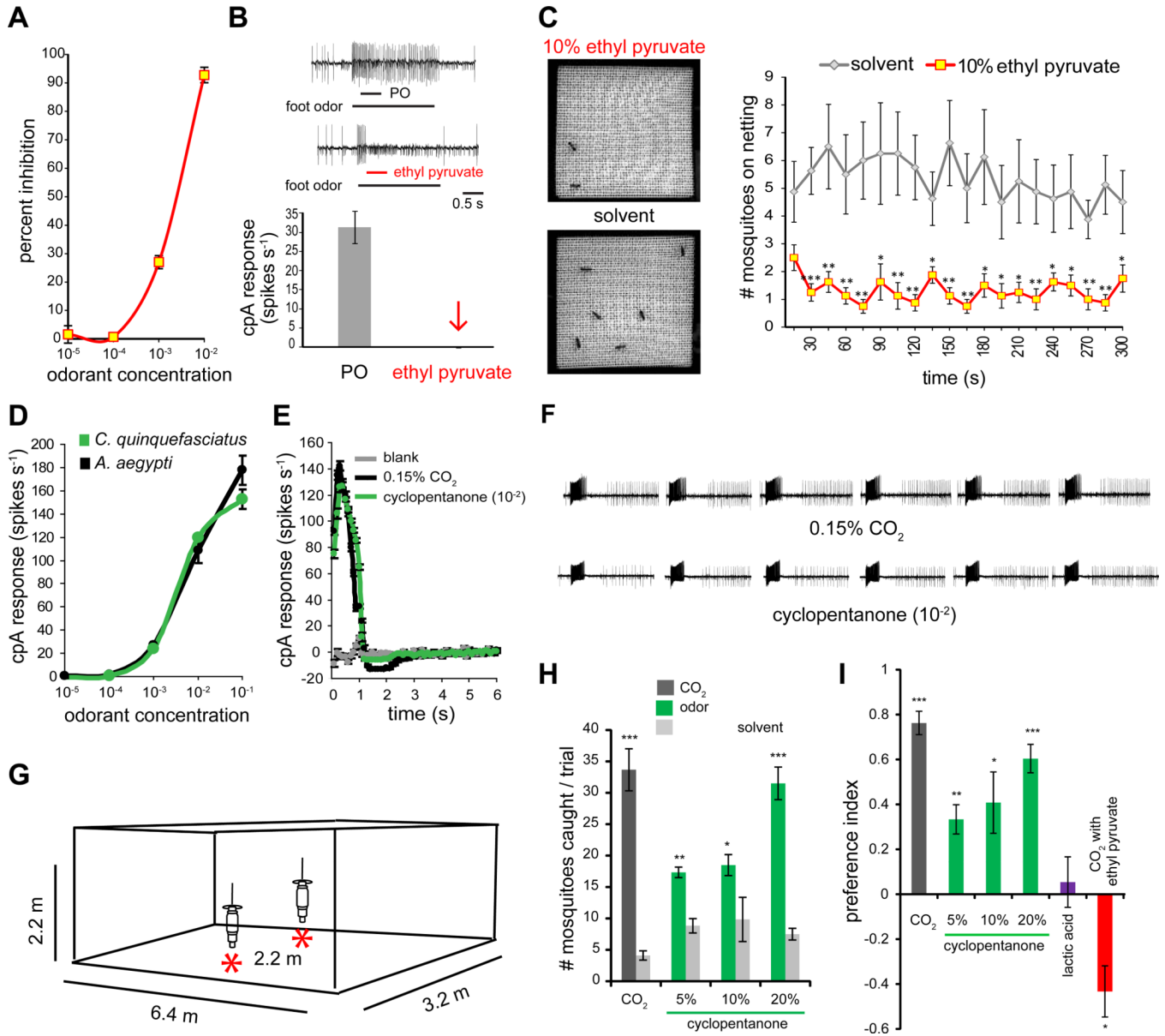
Figure 5. CO$_2$ receptor neuron inhibitors repel and activators attract mosquitoes
(**A**) Dose response of *A. aegypti* cpA neuron inhibition by a 1-s stimulus of ethyl pyruvate
(10^{-2}) overlaid on a 3-s stimulus of 0.15% CO$_2$. *n* = 6. (**B**) Representative trace and mean
response to a 1-s stimulus of ethyl pyruvate overlaid on a 2-s stimulus of foot odor (mixed
beads from Persons 1 and 2). *n* = 6. (**C**) Representative image of arm-in-cage mesh window
and mean number of mosquitoes on the netting over time for ethyl pyruvate–treated or
solvent-treated netting. *n* = 8. (**D**) Dose response of the cpA neuron to cyclopentanone in *A.
aegypti* and *C. quinquefasciatus*. *n* = 5–6. (**E**) Mean firing frequency during a 1-s stimulus,
counted in 100-ms bins during 1-s stimuli of cyclopentanone, CO$_2$, or blank odor cartridges.
Responses averaged across *n* = 4 replicates of 6 repeated stimuli in 20 s cycles. Total
baseline activity 5–6 s after each pulse was subtracted from response frequencies. (**F**)
Representative traces of repeated 1-s stimuli of cyclopentanone and 0.15% CO$_2$. (**G**)
Schematic of two-choice greenhouse experiments with two counterflow geometry traps. (**H**)
Mean number of mosquitoes captured out of 50 per trial in baited and control traps. *n* = 9

trials with CO_2, $n = 6$ for each cyclopentanone treatment. **(I)** Preference index for CO_2 and cyclopentanone trials (from **H)**, and for similar trials between lactic acid and solvent ($n = 6$) or between CO_2 and CO_2 with ethyl pyruvate ($n = 5$). t-test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Error bars are s.e.m. See also Figure S5.
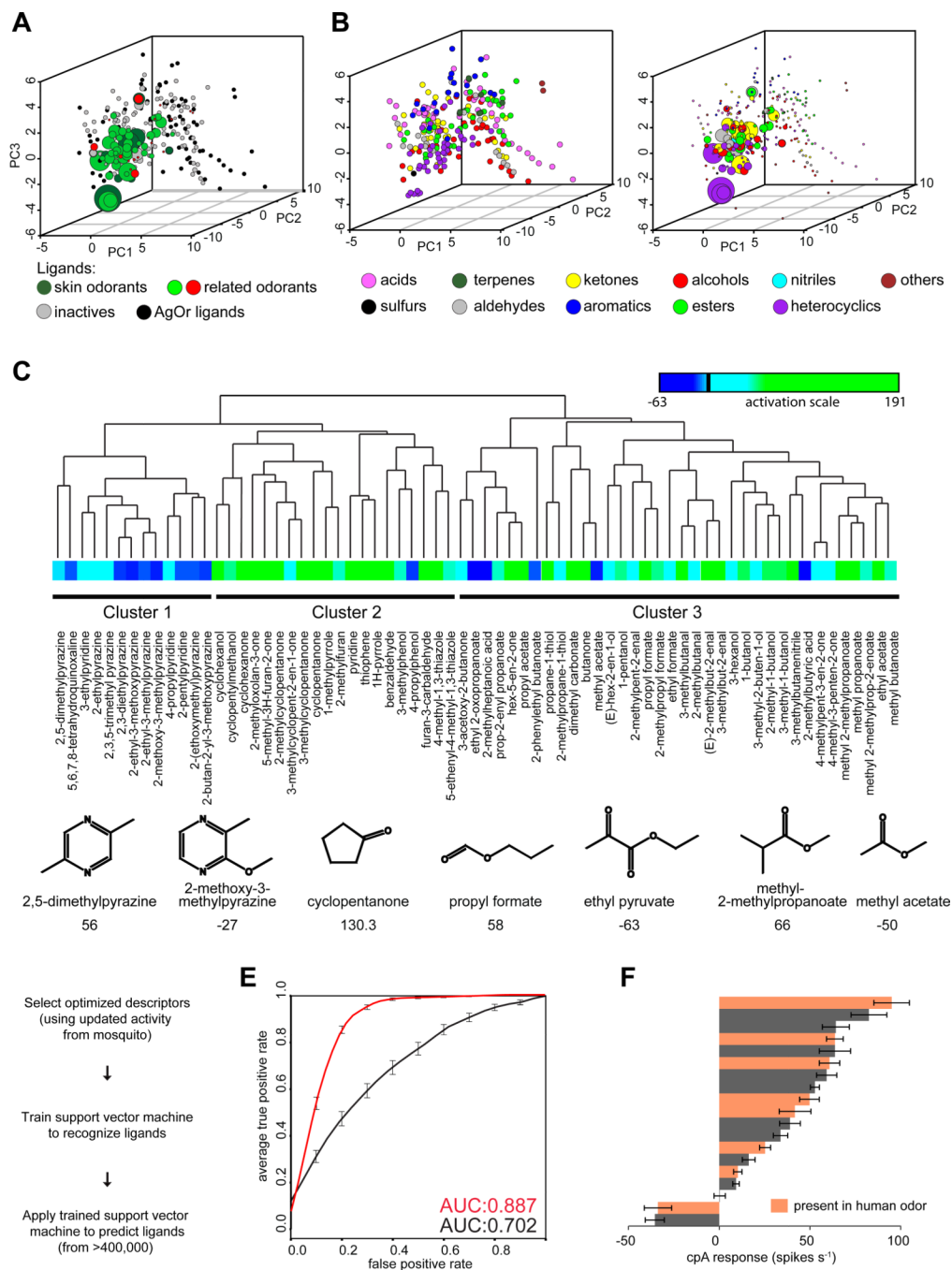
Figure 6. Encoding of chemical space by the mosquito cpA neuron
(A) Principle component analysis (PCA) of odorants calculated from 64 optimized molecular descriptor values. Circle size represents cpA activity evoked by each odorant. Dark green = human skin odorants; light green = predicted activators; red = predicted inhibitors; gray = predicted odorants that are inactive; black = odorants that activate *A. gambiae* olfactory receptors (AgOrs) (Carey et al., 2010). **(B)** The same analysis relabeled by chemical functional groups, with circle size representing cpA activity (right). **(C)** Hierarchical clustering and sample structures (with associated activity) of cpA-active odorants by activity-optimized descriptors. **(D)** Overview of the support vector machine

(SVM) integrated pipeline to improve computational prediction of novel CpA ligands. **(E)** Receiver-operating-characteristic (ROC) curve showing increased predictive accuracy of SVM method (red line) to our previous non-SVM method (black line) in a 5-fold cross-validation. **(F)** Mean responses of the *A. aegypti* cpA neuron to 0.5-s pulses of 19 newly predicted compounds screened as in Figure 4B. Salmon bars correspond to odorants found in human odor. $n = 4$. See also Tables S1–S2.
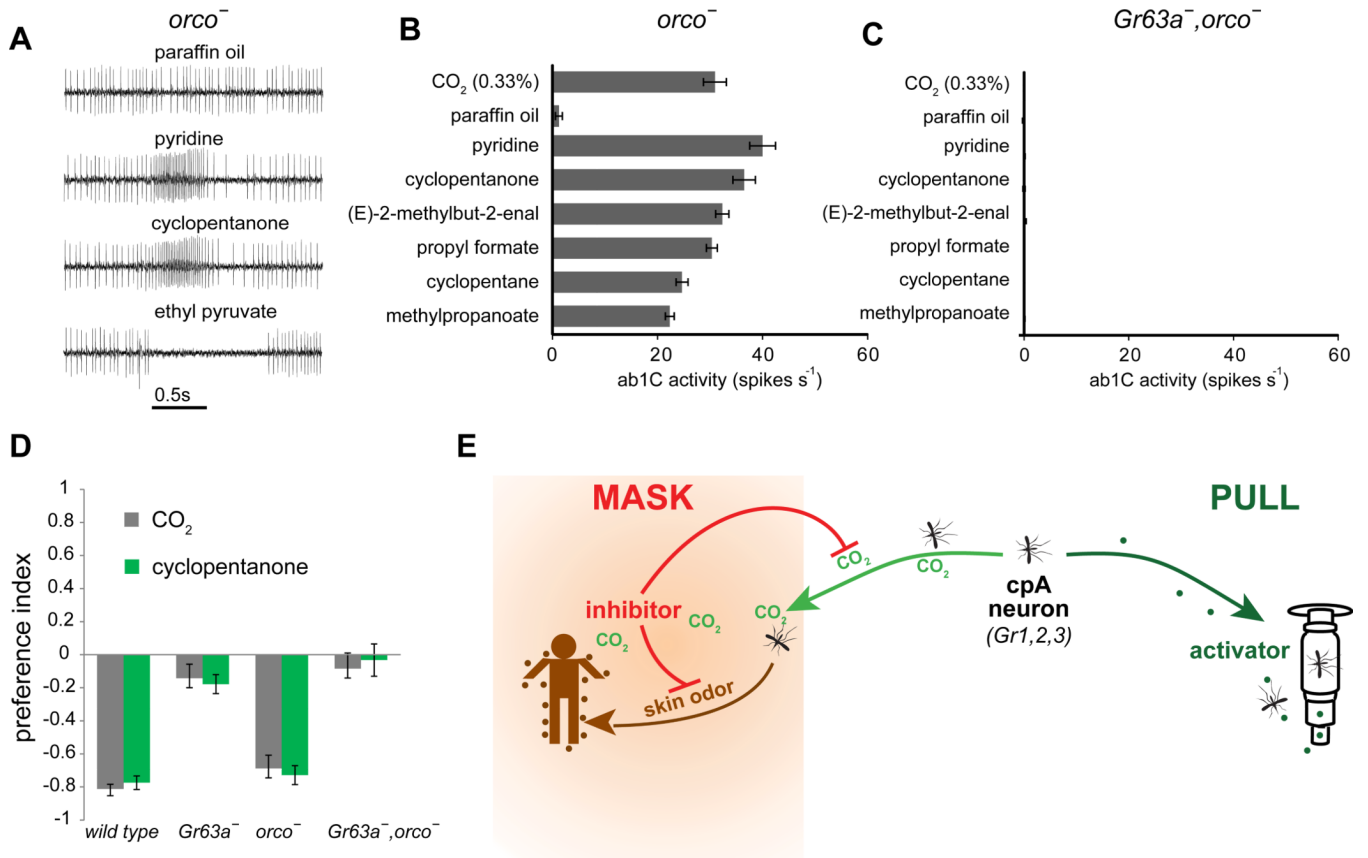
Figure 7. Detection of structurally diverse ligands depends on *Gr63a*
(**A**) Sample traces and (**B**) mean responses of the *Drosophila* ab1C neuron to a panel of odorants (tested in *orco⁻* flies). $n = 6$. (**C**) Mean responses from large basiconics in *Gr63a⁻,orco⁻* flies. $n = 24$ (6 sensilla per individual). (**D**) Mean preference index of flies of indicated genotypes to a choice between room air and 0.5% CO_2 or cyclopentanone (10^{-2}) in a T-maze. ($n = 12$–16). (**B–D**) Error bars are s.e.m. (**E**) The mosquito CO_2 receptor neuron plays a critical role in host-seeking by detecting both exhaled CO_2 and skin odor. Odor-based intervention strategies include use of inhibitors to block attraction to both CO_2 and skin odor (MASK) and activators as lures for traps (PULL). See also Figure S6.