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Moutaz Ali Agha

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Par

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Physiologie des récepteurs gustatifs chez la mouche de vinaigre
(*Drosophila melanogaster*)

Thèse présentée et soutenue à Gif sur Yvette, le 14 Décembre 2016 :

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ABREVIATIONS

Tableau 1 : Abréviations utilisées dans ce manuscrit

ADP	Adénosine diphosphate	Malt	Maltose
Arab	Arabinose	Mel	Melezitose
Ber	Berbérine	MultiCAFE	Dispositif d'observation du comportement alimentaire à capillaires multiples (Multi-Capillary Feeder)
Caff	Caféine	Nic	Nicotine
CS	souche de drosophile Canton Special	OR	Récepteur olfactif
Cyo	Curly oyster – mutation induisant une courbure aux ailes des drosophiles	ORN	Neurone récepteur olfactif
Den	Dénatonium	PER	Réponse d'extension du proboscis
DTI	toxine diphtérique	PR	Réponse de rétraction du proboscis
Fru	Fructose	PTX	Toxine Pertussi
GFP	Protéine fluorescente (green fluorescent protein)	SOG	Ganglion sous-oesophagien
Gluc	Glucose	Sorb	Sorbitol
Gly	Glycérol	TCC	Tricholine citrate
GPCR	Récepteur couplé à des protéines G	Theo	Théophiline
GR	Récepteur gustatif	Treh	Tréhalose
GRN	Neurone récepteur gustatif	UAS	Séquence activatrice (Upstream Activating Sequence)
Inos	Inositol	UMB	Umbelliferone
IR	Récepteur ionotropique	W¹¹¹⁸	souche de drosophile portant la mutation <i>white</i>
L-cana	L-canavanine		
LK	Leucokinine		
Lob	Lobeline		

I. Introduction générale

A. Introduction

Les êtres vivants, du plus petit au plus grand, sont capables de survivre à condition d'avoir la capacité de percevoir et d'interagir avec leur environnement pour se nourrir et se reproduire. Cette perception est assurée par un système nerveux et différents systèmes sensoriels comme les sens chimiques (gustation et olfaction), la vision et la mécano-réception (audition et proprioception). Les organes olfactifs et gustatifs sont particulièrement importants pour permettre aux animaux de bien évaluer et distinguer entre différentes ressources alimentaires, contenant des composés nutritifs ou des substances toxiques. Par exemple, lorsqu'une mouche se déplace sur un substrat, elle est capable de percevoir des odeurs par des récepteurs olfactifs localisés sur ses antennes et palpes labiaux, et des stimuli sapides ou peu volatils par l'intermédiaire de sensilles gustatives localisées sur ses pattes, ses pièces buccales et éventuellement ses ailes et son ovipositeur. Ces organes peuvent la renseigner sur la présence de substances toxiques ou irritantes par contact (qui poussent la mouche à quitter l'environnement toxique) ou d'odeurs induisant une répulsion à distance (qui permet à la mouche d'éviter le contact avec un pesticide par exemple). Les sens chimiques jouent un rôle tout particulier dans les relations entre les insectes et les plantes car ils permettent non seulement aux insectes de détecter des plantes propres à la consommation et à la survie de l'espèce, mais aussi d'éviter de consommer ou de s'approcher de plantes toxiques. Les sens chimiques jouent donc un rôle de sentinelle vis-à-vis de substances susceptibles d'entrer en contact direct avec l'organisme et ces substances sont extraordinairement variées.

B. Les plantes produisent une grande variété de molécules

Les molécules synthétisées par les plantes sont issues du métabolisme primaire ou secondaire. Le métabolisme primaire est défini comme concernant toutes les molécules essentielles à la vie, c'est-à-dire directement impliquées dans la croissance, le développement et la reproduction d'un organisme ou une cellule. Par définition, toutes les molécules qui ne sont pas directement impliquées dans ces processus fondamentaux sont considérées comme issues du métabolisme secondaire (Fraenkel, 1959). Pour les organismes qui ne sont pas autotrophes comme les plantes, il est essentiel de pouvoir localiser les molécules qu'ils sont incapables de synthétiser comme des sucres, des acides aminés, des lipides ou des stéroïdes. Ces molécules sont disponibles soit sous forme libre, soit sous forme condensée comme dans les organes de réserve des plantes. Une des fonctions des sens chimiques est donc de permettre aux organismes auxotrophes de localiser des sources d'éléments nutritifs et contribuer à estimer la valeur nutritive de ces ressources.

I.- Introduction générale

Les substances secondaires des plantes sont extrêmement variées. Ces substances sont très généralement considérées comme des substances de défenses contre les herbivores (Figure1), les pathogènes ou des compétiteurs, ou encore comme des adaptations à des conditions particulières (UV, froid, etc.) (Theis and Lerdau, 2003). Ces défenses sont constitutives et inducibles (Mithöfer and Boland, 2012). Leur nature chimique est extraordinairement diverse, allant de protéines de défense (Haruta et al., 2001), à large éventail de métabolites secondaires comme des alcaloïdes et des terpènes (Gershenzon, 1994; Purrington, 2000). Ces substances peuvent toucher des cibles communes à tous les êtres vivants (comme la respiration cellulaire avec les composés cyanogènes) ou plus spécifiques, comme les synapses cholinergiques (par exemple la nicotine). Pour les herbivores, ces composés peuvent non seulement être toxiques et affecter leur fitness mais aussi être anti-appétents ou répulsifs.

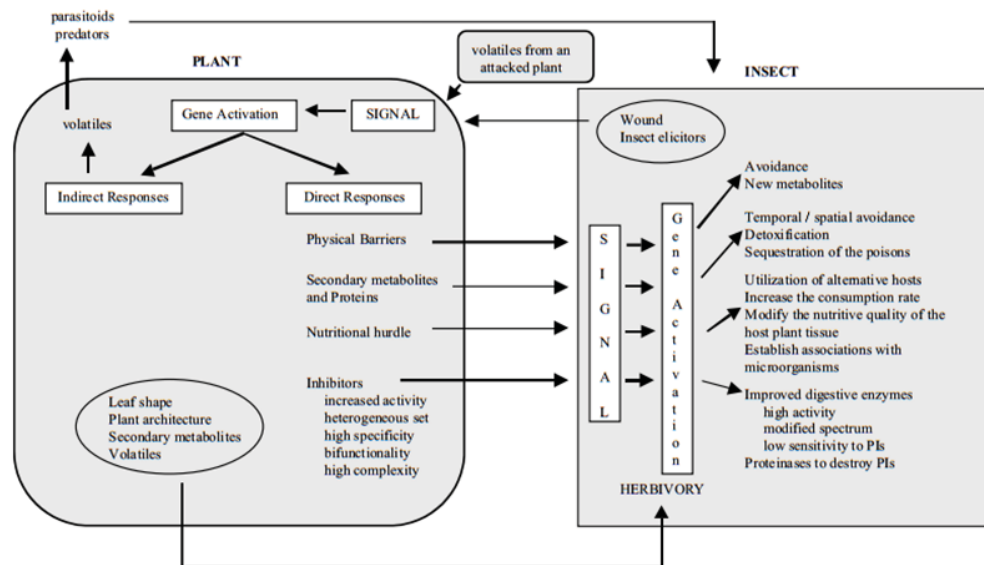


Figure 1 : Multiplicité des niveaux d'interactions entre les plantes et les insectes (Mello and Silva-Filho, 2002)

Certaines de ces substances sont connues par l'homme depuis longtemps. C'est le cas de la nicotine, qui est un alcaloïde soluble dans l'eau et produit à partir des feuilles de *Nicotiana tabacum L.*, utilisé à la fois comme un insecticide et comme un neurostimulant fortement addictif pour l'homme (Baselt, 2002). C'est le cas également de l'azadirachtine qui est le composé principal d'une huile extraite de graines d'un arbuste, *Azadirachta indica* (neem tree). Ce tri-terpénoïde est efficace contre beaucoup d'insectes avec une action anti-appétante et répulsive, mais aussi toxique et stérilisante (Isman, 2006). Cette substance perturbe la croissance des insectes à des doses très faibles (de l'ordre de 0.1 ppm). Les agriculteurs dans les pays en développement ont utilisé des formulations de graines de neem depuis de nombreuses années, principalement pour protéger les graines stockées.

Si les plantes présentent de nombreuses défenses chimiques contre les herbivores, les insectes qui les consomment utilisent une grande variété de mécanismes physiologiques et comportementaux pour contourner ces défenses (Brattsten, 1992). Les plus importantes sont des enzymes de détoxification de la famille des cytochrome P450 (Feyereisen, 1999). Une grande diversité de composés végétaux toxiques peuvent être métabolisés chez les insectes par des cytochromes P450 (Berenbaum et al., 1992). Par exemple, les solanacées produisent des composés secondaires qui les protègent des insectes (Harborne, 1986; Pomilio et al., 2008). Les larves de *Manduca sexta* qui colonisent les solanacées et en particulier le tabac, possèdent une enzyme P450 inducible par la présence de nicotine dans l'alimentation (Snyder and Glendinning, 1996; Stevens et al., 2000). Ces observations indiquent que les insectes s'adaptent à leur milieu alimentaire, ce qui pourrait être associé à des changements de sensibilité des neurones gustatifs périphériques. Actuellement, très peu de données existent sur ce sujet sauf sur *Manduca sexta*, où il aurait été montré qu'une adaptation physiologique à une plante hôte donnée induit des changements de sensibilité gustative (del Campo et al., 2001; Glendinning et al., 2001). Chez la drosophile également, nos collègues dijonnais montrent que l'exposition à de la caféine induit l'expression de P450 dans les tissus de l'insecte (Coelho et al., 2015). Il est possible que ces enzymes de détoxification puissent interférer avec la perception périphérique, à l'instar de P450 qui jouent un rôle dans la perception olfactive (Maibeche-Coisne et al., 2004).

Une de ces molécules a particulièrement retenu notre attention au cours de ce travail, c'est la L-canavanine, car elle semble évitée par un grand nombre d'insectes, dont la drosophile (Mitri et al., 2011). Cette molécule appartient à la classe des acides aminés non protéiques (Huang et al., 2011). Ils sont toxiques pour l'homme ainsi que pour l'animal domestique (Bell, 2003). La L-canavanine est accumulée à des concentrations très élevées dans les semences de plusieurs légumineuses (Bell, 1958, 1960; Rosenthal, 2001) et elle atteint par exemple 1,8 % de la matière sèche chez *Medicago sativa L.* (Rosenthal and Nkomo, 2000). La toxicité de cette molécule est due à sa structure qui est

proche de l'acide aminé L-arginine (Figure 2), ce qui lui permet d'interférer avec le métabolisme de l'arginine. Elle est incorporée au même titre que l'arginine dans les protéines nouvellement synthétisées par l'argininyl-tARN, donnant ainsi une conformation différente aux protéines ce qui généralement les rend inactives voire même toxiques (Rosenthal and Dahlman, 1975, 1986). Les propriétés insecticides de la L-canavanine ont été bien étudiées chez *Manduca sexta* (Rosenthal and Dahlman, 1975). D'après Rosenthal (2001), une mortalité larvaire apparaît chez *Manduca sexta* (L.) à des doses supérieures à 2,5 mM dans le milieu alimentaire, et 3% de la L-canavanine administrée est incorporée dans les protéines présentes dans l'hémolymphe 24 h après injection (Rosenthal et al., 1987).

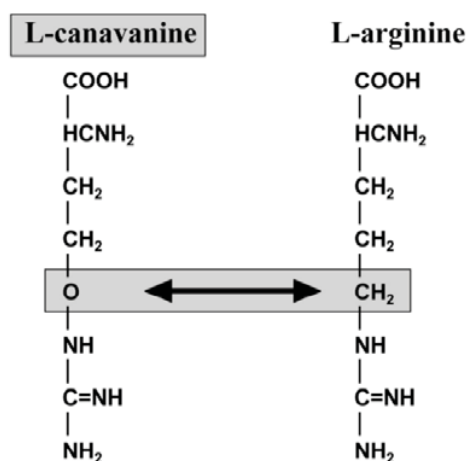


Figure 2 : Structures chimiques de la L-canavanine et la L-arginine montrant la différence de structure entre les deux composés (Mitri et al., 2009)

Cette toxicité n'est cependant pas universelle car certains insectes ont acquis la capacité de détoxifier cette molécule. C'est le cas de *Caryedes brasiliensis* (Coléoptère, Bruchidae), qui peut se nourrir sur les semences de *Dioclea megacarpa* Rolfe (Equisetopsida, Fabales) qui contiennent jusqu'à 8-9% de L-canavanine en poids sec. Cet insecte catabolise la L-canavanine en L-canaline et en urée (Rosenthal, 1977, 1983). *Heliothis virescens* (Lépidoptère, Noctuidae), qui est un ravageur très important pour les cultures, est également résistant à la L-canavanine car il est capable de la réduire en homosérine et hydroxyguantidine (Bell, 1958, 1960 ; Rosenthal, 2001). Cependant, bien que *H. virescens* soit résistant à la L-canavanine, les larves de cet insecte détectent ce composé (Simmonds et al., 1990).

En résumé, les plantes produisent une grande diversité de molécules. Certaines de ces molécules ont une valeur nutritionnelle alors que d'autres représentent un danger pour le consommateur. Les herbivores et tout particulièrement les insectes, ont développé des sens chimiques qui leur permettent de caractériser ces plantes à partir des molécules odorantes ou

sapides qu'elles émettent. Les odeurs de plantes font l'objet d'une attention toute particulière depuis de nombreuses années, parce qu'elles portent la signature de l'espèce de plante et de son état physiologique, non seulement pour des insectes herbivores ou pollinisateurs, mais aussi pour des insectes prédateurs ou parasites de ceux-ci (Bruce et al., 2005). Les composés non volatils peuvent également être caractéristiques de l'identité spécifique des plantes (Dethier, 1980), mais aussi de leur valeur nutritive, en particulier pour les organes de réserve. Parmi ces composés non-volatils, il existe donc toute une série de composés sapides comme les sucres ou les acides aminés qui servent de signal à une alimentation nutritive, et un grand nombre de molécules qui sont toxiques ou qui signalent la présence de substances toxiques.

Comment une telle multiplicité de molécules et de mélanges complexes est-elle détectée et analysée par les insectes phytophages ? C'est cette question qui sous-tend les études rassemblées dans ce manuscrit. L'exemple de la L-canavanine illustre le fait que les composés secondaires des plantes n'ont pas une valeur de protection universelle et que les insectes peuvent utiliser différentes stratégies pour détoxifier certaines de ces molécules afin d'utiliser ces plantes. Ces adaptations ont été considérées comme un élément central de la coévolution plantes-insectes (Ehrlich and Raven, 1964). Alors que très généralement, les signaux aversifs ou répulsifs des plantes signalent un danger comme la présence de composés toxiques pour les herbivores, pour les espèces d'insectes adaptées, ces signaux peuvent au contraire signaler la présence de plantes hôtes. Ces espèces acquièrent des capacités de détection de signaux spécifiques qui les aident à coloniser ces plantes. Un exemple particulièrement illustratif à cet égard est constitué par *Drosophila sechellia* qui est capable de coloniser un fruit normalement toxique pour les drosophiles, *Morinda citrifolia* (Rkha et al., 1991). Cette espèce de drosophile a divergé très récemment de l'espèce *Drosophila simulans* qui n'accepte pas *Morinda* pour se développer. *D. sechellia* a non seulement acquis des capacités de tolérance à l'acide octanoïque présent dans ces fruits mais les adultes possèdent des modifications de leur système olfactif et gustatif qui leur permettent de pondre sur ces fruits (McBride, 2007; Harada et al., 2008; Kopp et al., 2008; Stensmyr, 2009).

Il n'en reste pas moins que tous les insectes phytophages possèdent un système gustatif capable de détecter des molécules aversives, provenant de leur environnement, par exemple de plantes non-hôtes ou d'organismes avec lesquels ils sont en compétition comme des champignons ou bactéries, ou encore provenant de parasites (French et al., 2015a). Dans ce travail, nous avons principalement examiné comment ces composés gustatifs aversifs pour l'espèce modèle, *Drosophila melanogaster*, sont détectés lorsqu'ils sont présents en mélange avec des molécules attractives comme des sucres, en nous focalisant sur la réponse de neurones gustatifs répondant à des substances amères ou bien à des sucres.

C. Le système gustatif détecte des catégories de molécules sapides

A priori, chez l'homme, le mot «goût» est utilisé pour décrire les sensations découlant de la cavité buccale. Ces sensations comprennent la gustation proprement dite, *i.e.* les sensations induites par un réseau de cellules sensorielles spécialisées, regroupées dans les papilles gustatives de la cavité buccale, et ainsi que d'autres sensations comme des odeurs, détectées par le système olfactif rétronasal, des sensations mécano-sensorielles lors de la mastication, des sensations de température (chaude, froide) et des sensations d'irritation ou de brûlure. Les composants non gustatifs du goût sont détectés par des systèmes sensoriels différents de la gustation, l'olfaction rétro-nasale pour les odeurs, et le système somesthésique pour les sensations mécaniques (Bachmanov and Beauchamp, 2007). Les insectes possèdent également un système gustatif, mais il est constitué d'unités sensorielles indépendantes appelées sensilles.

1. Organisation du système gustatif de la drosophile

Drosophila melanogaster est un insecte qui appartient à la famille Drosophilidae de l'ordre des Diptères. Comme chez d'autres insectes, les cellules gustatives sont insérées dans des unités sensorielles appelées sensilles, qui sont non seulement présentes sur les pièces buccales, mais aussi sur les pattes, les ailes et l'ovipositeur (Stocker, 1994; Montell, 2009) ou encore sur les antennes chez les Lépidoptères par exemple. Les sensilles gustatives se différencient des sensilles olfactives ou mécano-sensorielles par un pore terminal (Figure 3). C'est par ce pore terminal que les molécules de l'extérieur pénètrent dans la sensille, et entrent en contact avec les dendrites des neurones gustatifs. Le corps cellulaire de ces neurones est inséré dans la couche de cellules épithéliales située sous la cuticule, et entouré de cellules accessoires qui participent à la mise en place de l'appareil cuticulaire de la sensille et ensuite se différencient en cellules sécrétrices. Associés à ces neurones gustatifs, à la base des sensilles, on trouve le plus souvent un neurone mécanorécepteur.

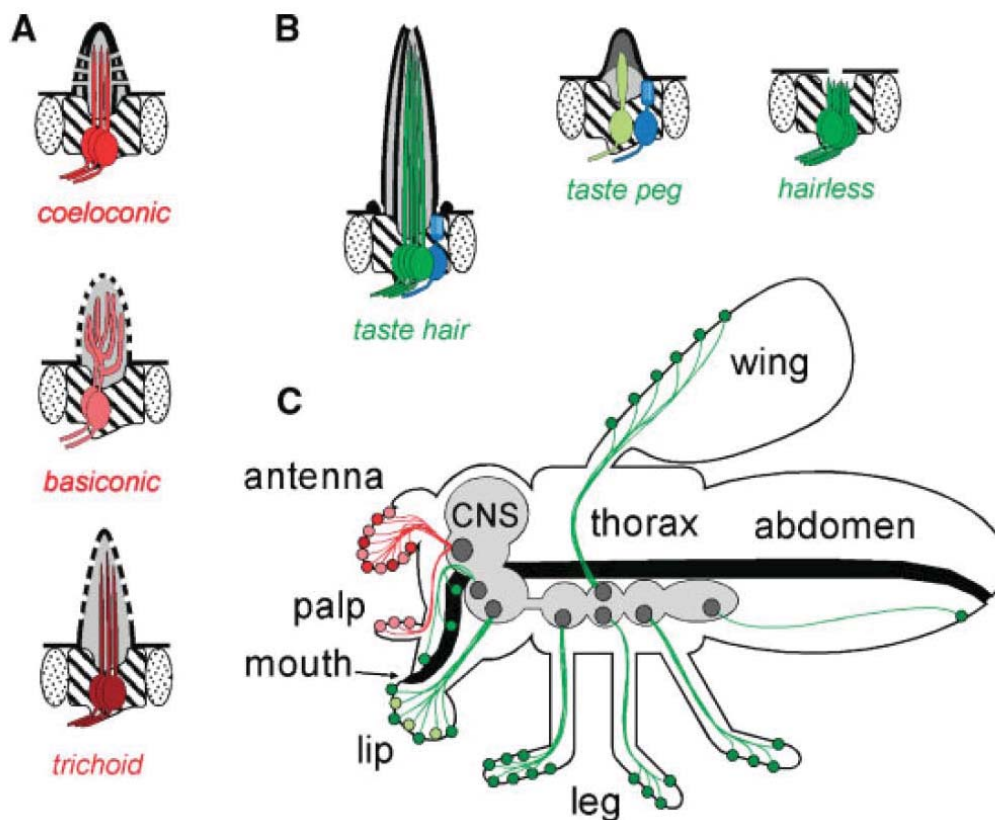


Figure 3 : Distribution des sensilles chimiosensorielles chez *D. melanogaster* d'après de Bruyne and Warr (2006)

A. Types de sensilles olfactives. Selon la forme de l'appareil cuticulaire et la disposition des neurones, on distingue trois types de sensilles olfactives : coeloconiques, basiconiques et trichoïdes. La caractéristique commune de ces sensilles est que la cuticule de la soie est percée d'une multitude de petits pores par lesquels les molécules odorantes pénètrent et parviennent au contact des dendrites des neurones olfactifs. Les neurones olfactifs (figurés en rouge) sont bipolaires et envoient leur axone vers le cerveau, tandis que leur dendrite simple ou divisé baigne dans la cavité de la soie. Ces neurones sont entourés de cellules épithéliales accessoires (hachures) qui élaborent le liquide sensillaire dans lequel baignent les dendrites de ces neurones. **B** – Les sensilles gustatives sont constituées sur le même schéma (appareil cuticulaire, neurones bipolaires et cellules accessoires) mais se distinguent des sensilles olfactives par la présence d'un pore à leur extrémité. Selon la taille de leur appareil cuticulaire, on distingue des sensilles gustatives trichoïdes (taste hair), des papilles gustatives externes (taste peg) et des papilles gustatives internes sans appareil cuticulaire (hairless). Ces sensilles gustatives comprennent des neurones gustatifs (4, 2 ou 1 – en vert) et pour les sensilles externes, un neurone mécanorécepteur (en bleu). **C** – Schéma présentant la distribution des sensilles gustatives (en vert) et des sensilles olfactives (en rouge) à la surface du corps des insectes. Le système nerveux central est représenté en gris et le tube digestif en noir. Les neurones olfactifs des antennes et des palpes maxillaires convergent dans le lobe antennaire. Les neurones gustatifs envoient leurs axones dans le segment ipsilatéral, certains d'entre eux poursuivant leur chemin pour rejoindre le ganglion sous-oesophagien.

Les sensilles que nous avons le plus étudié dans notre travail sont les sensilles situées sur le proboscis. Celui-ci comporte 31 sensilles trichoïdes situées de chaque côté du labellum ainsi qu'un nombre équivalent de sensilles gustatives intertrachéales (Stocker, 1994). Les sensilles du premier type sont organisées en quatre lignes orientées selon un axe antéro – postérieur. Ces sensilles ont

été classifiées en 3 types, sur la base de leur taille : longues (L), intermédiaires (I) et courtes (s) (Shanbhag et al., 2001).

Ces sensilles ont été numérotées en partant de la position antérieure du labellum afin de les cartographier (Hiroi et al., 2002). La position de ces sensilles est relativement stable : aucune variation n'a été trouvée dans le nombre total de sensilles de type L chez des femelles de souche Canton S ; par contre de petites variations ont été observées dans le nombre total de sensilles de type S (S: 12 – 13) ainsi que de type I (I : 9 – 10) (Hiroi et al., 2002)(Figure 4-E). Récemment, ce schéma a été modifié par l'équipe de Carlson en 2011 (Figure 5).

Une autre classification de sensilles du proboscis a été proposée par Weiss et al. (2011) sur la base de critères fonctionnels, en fonction de la distribution des différents Grs dans ces sensilles et de leur capacité à répondre à des substances amères. Selon ces critères, les sensilles se distribuent en 5 classes (Figure 6).

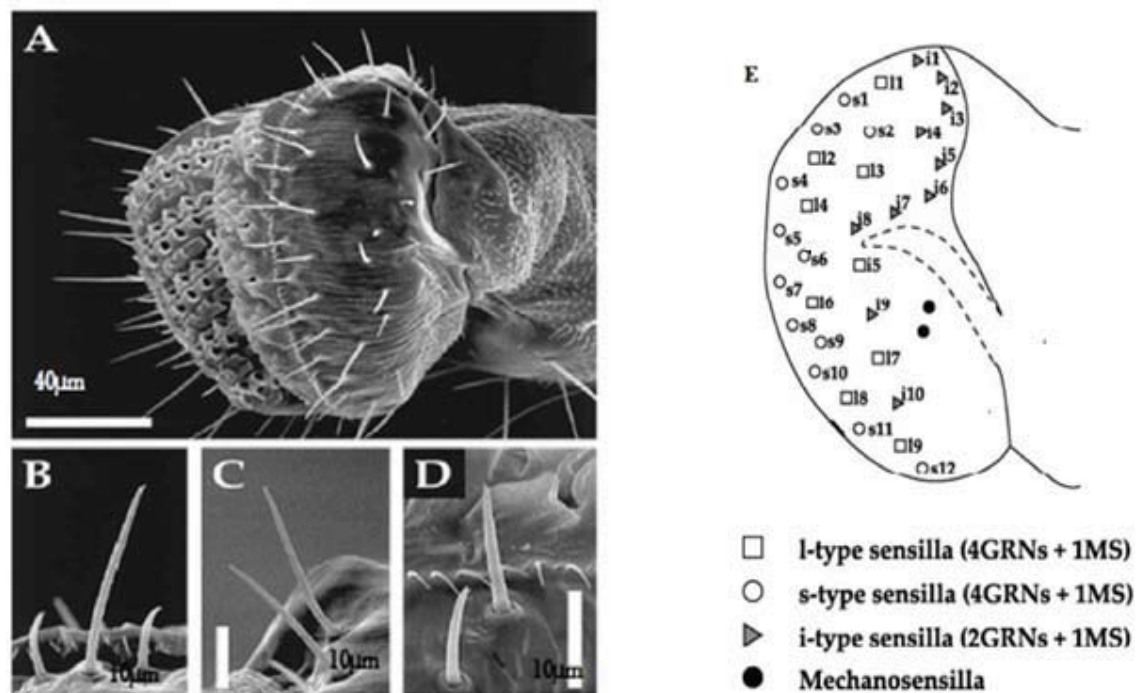


Figure 4 : Sensilles gustatives du proboscis de drosophile (Hiroi et al., 2002)

(A) Vue latérale du lobe gauche du labellum. (B) sensille type L. (C) Type S. (D) type I. (E) Implantation des sensilles.

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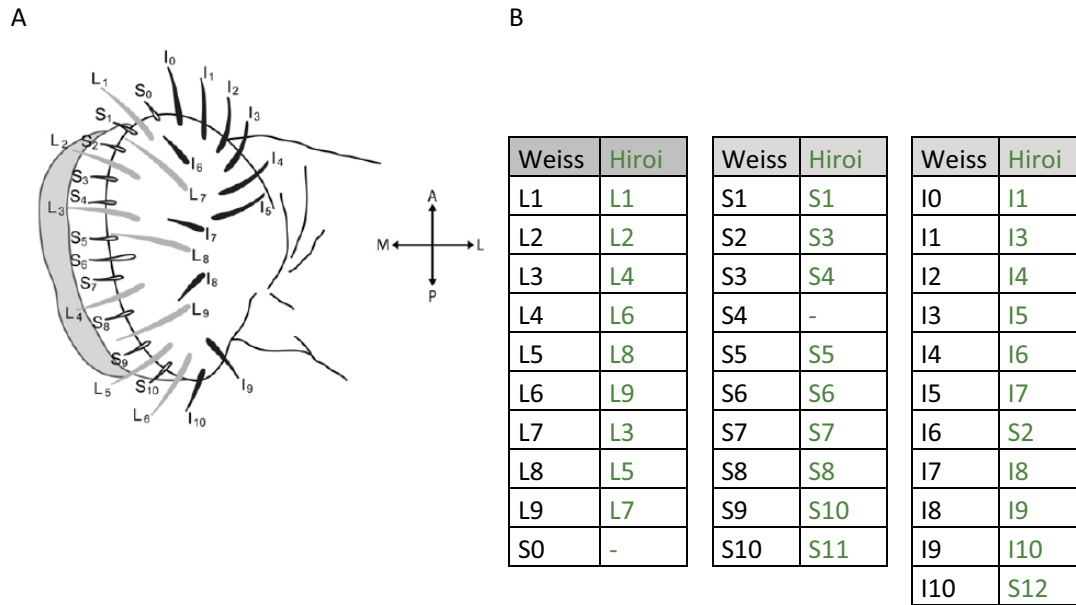


Figure 5 : Cartographie des sensilles, modifiée par Weiss et al. (2011)

(A) Diagramme d'implantation des sensilles du proboscis. (B) Tableau de correspondance entre les deux nomenclatures

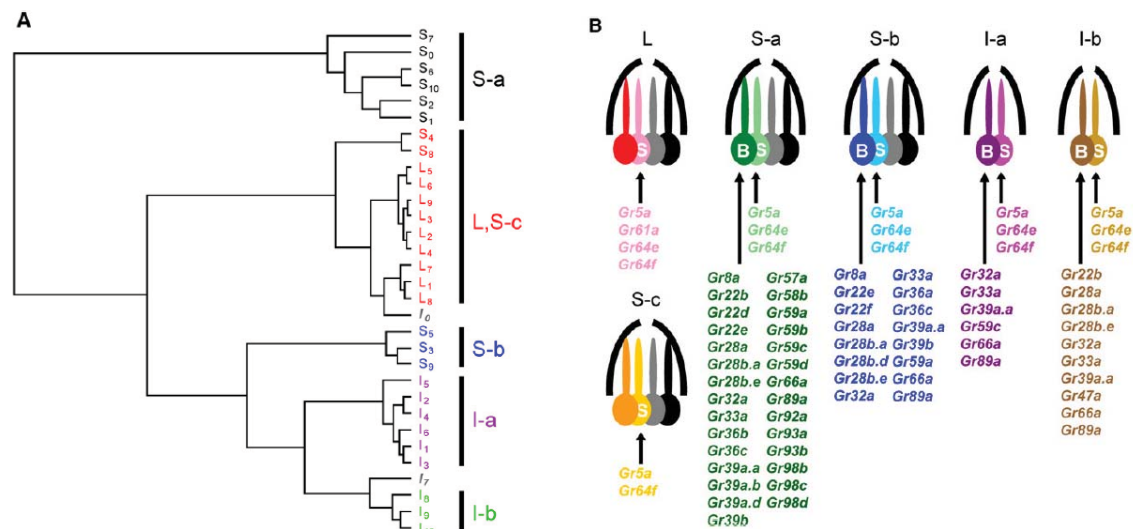


Figure 6 : Classes fonctionnelles de sensilles gustatives sur le proboscis de drosophiles (Weiss et al., 2011)

(A) Groupement des sensilles du proboscis en classes fonctionnelles sur la base de leurs profils de réponses à un échantillon de 16 substances amères. (B). Co-expression de Grs dans les neurones répondant à l'amer (« B ») et dans les neurones répondant aux sucres (« S »), en fonction du type sensillaire.

2. Physiologie des neurones gustatifs de la drosophile

Les neurones gustatifs des insectes expriment des récepteurs membranaires spécifiques. La plus importante est une famille de protéines à 7 domaines transmembranaires, les récepteurs gustatifs (Grs) (Clyne et al., 2000) qui appartiennent à la même classe que les récepteurs olfactifs d'insectes (Ors) (Robertson et al., 2003). Soixante Grs ont été identifiés chez la Drosophile, et ils encodent un total de 68 protéines réceptrices (Robertson et al., 2003). Ils sont exprimés dans les neurones gustatifs externes (Clyne et al., 2000; Dunipace et al., 2001; Isono and Morita, 2010). Les Grs sont exprimés également dans d'autres tissus comme des cellules du tube digestif (Park and Kwon, 2011), mais aussi dans l'antenne où ils participent à la thermoréception (Ni et al., 2013), dans les neurones olfactifs exprimant des ORs (où leur rôle est inconnu) ou dans des cellules du cerveau où ils participent à la régulation de la glycémie (Fujii et al., 2015). Enfin, deux récepteurs gustatifs (Gr21a et Gr63a) sont impliqués dans la détection du CO₂ et sont exprimés dans des sensilles olfactives de l'antenne (Jones et al., 2007; Kwon et al., 2007).

Les sensilles gustatives de drosophiles expriment également d'autres familles de molécules directement ou indirectement impliquées dans la transduction et dans la détection des molécules sapides. Comme les neurones olfactifs où ils ont été initialement décrits (Benton et al., 2009), les neurones gustatifs expriment des récepteurs « ionotropiques » (IRs) (Croset et al., 2010) qui ont été impliqués dans la détection de phéromones (Koh et al., 2014; Stewart et al., 2015), de sels (Zhang et al., 2013) ou de polyamines (Hussain et al., 2016). Outre ces IRs, les neurones gustatifs expriment également des canaux ioniques de la famille des TRPs, qui, chez les vertébrés, sont impliqués dans la détection de la température, du toucher, de la douleur, de l'osmolarité, des phéromones, du goût et d'autres stimuli (Clapham, 2003). Chez les insectes, TRPA est impliqué dans la détection du wasabi et de molécules « piquantes » (Al-Anzi et al., 2006). TRPA1 est impliqué dans la détection de substances répulsives comme l'acide aristolochique (Kim et al., 2010) mais aussi dans la réponse à des stimuli nociceptifs comme la température (Kim et al., 2010), ou dans la réponse à des substances répulsives chez le moustique (Sang Hoon, 2013; Du et al., 2015). Enfin, des canaux ioniques de la famille « Pickpocket » initialement découverts dans le cadre de la mécanoréception (Adams et al., 1998), sont impliqués respectivement dans la détection de l'eau (ppk28 : Cameron et al., 2010; Chen et al., 2010; Waterson et al., 2014), ou de sels (ppk11 : Liu et al., 2003) ou de phéromones sexuelles (ppk23, ppk25 : Lu et al., 2012; Toda et al., 2012; Vijayan et al., 2014). Outre ces molécules directement impliquées dans la détection de stimuli chimiques ou thermiques, les neurones gustatifs sont environnés de cellules épithéliales accessoires qui participent à la bonne détection des molécules, notamment en sécrétant des protéines de transport comme des « odorant binding proteins » (OBP) qui sont impliquées notamment dans la détection des substances amères (Jeong et al., 2013) ou de

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molécules spécifiques de la plante hôte (Matsuo et al., 2007), et des protéines de transport appelées CheB qui sont impliquées dans la détection de phéromones sexuelles (Xu et al., 2002; Park et al., 2006; Starostina et al., 2009). D'autres gènes sont également impliqués dans la détection de substances particulières comme détection de sels comme *Serrano* (Alves et al., 2014).

En résumé, on connaît un grand nombre de gènes qui sont exprimés dans les neurones gustatifs de drosophile. Bien que la structure cellulaire et nombre de récepteurs soient partagés par les neurones gustatifs et les neurones olfactifs, chaque neurone gustatif possède une identité fonctionnelle liée à la co-expression de plusieurs récepteurs gustatifs (jusqu'à 29 pour les neurones sensibles à l'amer) alors que les neurones olfactifs sont caractérisés par l'expression d'un seul récepteur olfactif comme chez les vertébrés (French et al., 2015a). C'est la diversité des mosaïques de récepteurs exprimés dans chaque neurone qui est sans doute responsable des types de réponses différentes que l'on observe entre sensilles selon leur implantation (Meunier et al., 2003b), et qui a permis à Weiss de définir des types fonctionnels sur le proboscis (Weiss et al., 2011).

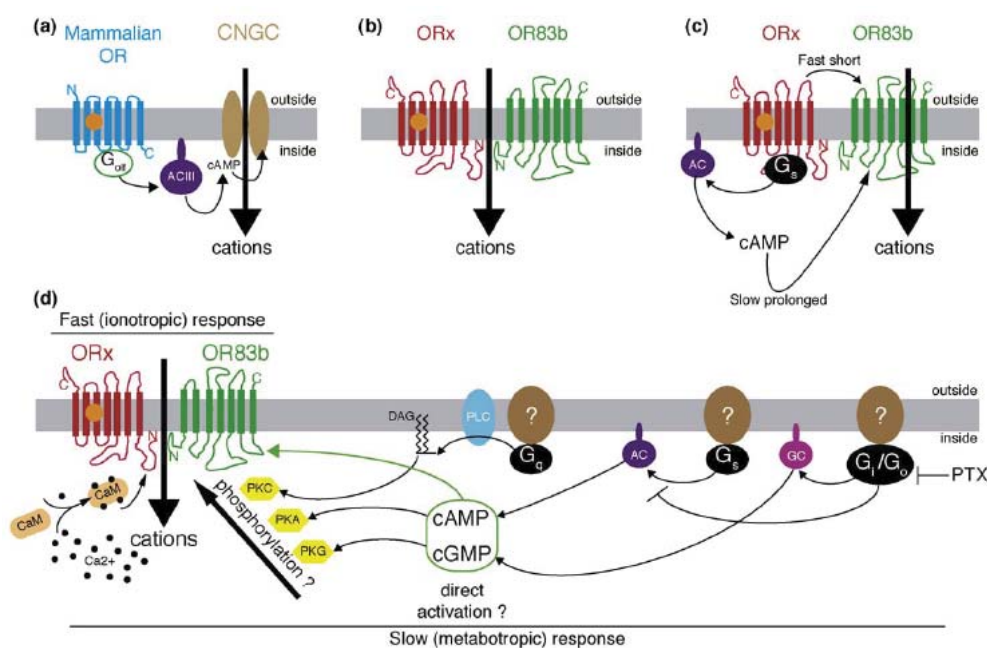


Figure 7 : Modèles actuels de la transduction olfactive chez les insectes selon Nakagawa and Vosshall (2009)

Dans cette figure, les auteurs résument les principales hypothèses actuelles concernant la transduction olfactive observée lorsqu'un ligand s'associe à un récepteur protéique membranaire de type OR. (a) Transduction canonique qualifiée de métabotrope chez les Vertébrés, où le ligand induit une association entre le récepteur avec des protéines G, ce qui libère de l'AMPc qui va ouvrir des canaux AMPc dépendants. (b) Transduction par ouverture de pore ionique directement, appelée ionotrope (Sato et al., 2008). (c) Transduction par ouverture d'un pore ionique et libération d'AMPc qui activerait Orco (ici : OR83b) (Wicher et al., 2008). (d) Modèle proposé par Nakagawa et Vosshall pour intégrer les deux modalités, incluant une activation d'Orco par l'AMPc. Dans ce schéma, l'activation du récepteur ORx induit une réponse rapide ionotrope et brève, et une activation métabotrope lente et prolongée. La cascade métabotrope implique un couplage entre plusieurs éléments membranaires non identifiés et plusieurs protéines G, ce qui induit la production d'AMPc et de GMPc intracellulaires qui activent secondairement Orco.

Alors que les protéines réceptrices du goût et de l'olfaction commencent à être bien connues, les voies de transduction liées à l'activation de ces protéines sont encore incomplètement décrites. Jusqu'à très récemment, on considérait que les récepteurs des insectes fonctionnaient comme les récepteurs des Vertébrés, c'est-à-dire comme des récepteurs liés à des protéines G (Keller and Vosshall, 2003). Benton et al. (2006) ont montré que l'insertion de ces récepteurs dans la membrane cellulaire était inversée par rapport à ce qui est trouvé chez les vertébrés. Cette topologie inversée, confirmée indépendamment dans le groupe de J. Carlson (Lundin et al., 2007), implique que les sites du récepteur susceptibles d'interagir avec les protéines G sont extracellulaires. En d'autres termes, l'activation des récepteurs olfactifs (et gustatifs) d'insectes par un ligand n'activerait pas de cascade de transduction liée à des protéines G. L'hypothèse dominante actuellement est que ces récepteurs (dimériques au moins dans le cas de l'olfaction), agiraient comme un pore ionique (Sato et al., 2008). Une seconde hypothèse est que ces récepteurs agiraient à la fois comme un pore ionique et comme un canal lié à des protéines G (Wicher et al., 2008). Ces deux hypothèses coexistent actuellement sans parvenir à un consensus qui soit universellement accepté (Nakagawa and Vosshall, 2009) (Figure 7).

Compte tenu de la relation de proximité phylogénétique entre récepteurs olfactifs et gustatifs d'insectes, il est probable que les récepteurs gustatifs fonctionnent selon les mêmes modalités. Deux travaux au moins confirment l'insertion « inversée » des récepteurs gustatifs dans les membranes chez des insectes de la famille des lépidoptères (Zhang et al., 2011; Xu et al., 2012). Certaines protéines gustatives réceptrices sont considérées comme pouvant former des canaux récepteurs homo-dimériques comme *BmGr9* et *DmGr43a* (Sato et al., 2011), mais l'hypothèse généralement admise est que les récepteurs gustatifs forment des multimères entre plusieurs GRs (Slone et al., 2007).

3. Rôle des différentes sensilles et représentation centrale des informations gustatives

Les cellules gustatives des drosophiles sont dédiés à la détection d'une large palette de composés chimiques (Thorne et al., 2004; Scott, 2005; Yarmolinsky et al., 2009). Le rôle des sensilles gustatives dépend de leur position sur la surface du corps. Les sensilles présentes sur le proboscis et sur les pattes leur permettent d'évaluer la nourriture avant ingestion, mais également à identifier le sexe d'un partenaire au cours de la rencontre des sexes. Chez les mâles de drosophiles, les sensilles situées sur la première paire de pattes sont plus nombreuses que chez les femelles (50 versus 37), ce qui leur permet de détecter les phéromones non volatiles qui favorisent l'accouplement avec des femelles (Bray and Amrein, 2003). Alors que le fonctionnement et le rôle des sensilles externes du proboscis et des pattes commence à être bien connu, les papilles gustatives du proboscis (« taste

pegs ») et les papilles du tube digestif antérieur (hairless) sont moins accessibles. Récemment, toute une série de travaux utilisant des marqueurs génétiques spécifiques des sensilles du tube digestif antérieur ont permis de montrer que ces sensilles ont un rôle crucial dans la prise de nourriture et les choix alimentaires (LeDue et al., 2015; Yapici et al., 2016).

Les axones des neurones gustatifs se projettent dans le ganglion nerveux correspondant à l'appendice sur lequel ils sont insérés, soit au niveau du ganglion sous-œsophagien pour les pièces buccales, soit au niveau des ganglions thoraciques pour les sensilles gustatives situées sur les pattes ou les ailes. Bien que la majorité des neurones gustatifs se projette dans le ganglion ipsilatéral, une partie d'entre eux se projette également dans le ganglion sous-œsophagien.

Contrairement au système olfactif dont le premier relai synaptique est organisé en glomérules correspondant aux projections de neurones olfactifs exprimant le même récepteur, les aires du système nerveux central dans lesquelles les axones des neurones gustatifs périphériques se terminent (appelées aires de projection) sont plus diffuses. Ces aires de projection comportent une dimension somatotopique, en fonction de la place des soies sur l'appendice correspondant (Newland et al., 2000). On admet généralement que les projections des neurones gustatifs se distribuent également au moins en deux zones, l'une correspondant à des neurones répondant aux sucres et l'autre à des neurones répondant aux stimuli amers (Wang et al., 2004). Ces deux modalités principales étaient déjà postulées chez les insectes phytophages par des auteurs antérieurs sur la base de l'analyse des réponses électrophysiologiques (Chapman, 2003) et de leur correspondance avec la stimulation ou l'inhibition du comportement alimentaire. Une des raisons qui peuvent expliquer cette organisation moins « claire » que le système olfactif est que le système gustatif intervient non seulement dans des comportements et des choix alimentaires, mais aussi induit des réactions hormonales ou sécrétrices, en particulier au niveau du tube digestif comme cela a été montré chez les larves de drosophiles (Melcher and Pankratz, 2005).

En tout état de cause, chez les insectes comme maintenant chez les vertébrés, les données moléculaires suggèrent que le système gustatif n'encode pas les informations selon un schéma de type « across fiber pattern » (Erickson, 2008) mais plutôt sous la forme de classes de neurones récepteurs comme le sucré et l'amer (Barretto et al., 2015). Ce principe de codage (schématisé dans la Figure 8) simplifie le décodage des informations par le système nerveux central mais limite le nombre de catégories susceptibles d'être discriminées par le système (Masek and Scott, 2010). Cette vision simplificatrice ne fait cependant pas l'unanimité mais nous manquons de tests comportementaux pour valider ou infirmer cette hypothèse chez les insectes, en particulier en ce qui concerne la détection des substances amères car les tests d'apprentissage reposent sur des stimulations mixtes (sucre + amer).

Codage de type « labeled lines »	Neurone classe 1	Neurone classe 2	Neurone classe 3	Neurone classe 4
Ligands A (1..n)	++	0	0	0
Ligands B (1..n)	0	++	0	0
Ligands C (1...n)	0	0	++	0
Ligands D (1...n)	0	0	0	++

Codage de type « across fiber »	Neurone classe 1	Neurone classe 2	Neurone classe 3	Neurone classe 4
Ligands A (1..n)	0	++	0	++
Ligands B (1..n)	++	+	0	++
Ligands C (1...n)	+	0	++	0
Ligands D (1...n)	++	0	0	++

Figure 8 : Principe du codage "across fiber" et "labeled lines"

En gustation, très souvent deux types de codage nerveux sont évoqués. Le codage de type « labeled lines » correspond à des neurones qui sont excités par un seul composé ou une seule classe de composés. Ce type de codage est classique pour la détection des phéromones. En gustation, il correspond à une identité de chaque neurone. Quand les neurones d'une classe donnée sont actifs, c'est qu'ils sont activés par un ligand d'une classe et pas par d'autres ligands. Le codage de type « across fiber pattern » correspond à des activations non sélectives, chaque neurone pouvant répondre à plusieurs types de ligands. La seule manière d'identifier un stimulus est de comparer les réponses de différentes classes de neurones. Ici, les classes de neurones sont représentées sous formes de colonnes de différentes couleurs. Les classes de ligands sont disposées en lignes successives. L'activation des neurones est indiquées par le signe « ++ » et l'absence d'activité par « 0 ».

4. La Drosophile dispose d'outils génétiques puissants

La drosophile fait l'objet d'études génétiques très poussées qui ont été rendues possibles ou facilitées par un nombre de facteurs. Très tôt, la communauté des drosophilistes s'est mobilisée pour mettre en commun des outils, notamment des mutants et des constructions génétiques, ce qui a abouti à la création de plusieurs « banques » de mutants, accessibles à tous les chercheurs, et aussi à la mise en place de plusieurs bases de données génomiques et génétiques accessibles en ligne, comme Flybase¹. Parallèlement, les généticiens de la drosophile ont imaginé et mis en place un système extrêmement original permettant de conserver des mutations ponctuelles en les associant à des mutations visibles grâce à des marqueurs phénotypiques (comme *white* correspondant à des yeux blancs), associations qui sont maintenues de manière durable au cours des générations successives grâce à la création de chromosomes balanceurs qui ont été générés pour chaque chromosome. Ces chromosomes empêchent la ségrégation des caractères associés par recombinaison, grâce à 3 propriétés : (1) ils portent des inversions qui ne permettent plus d'appariement et limitent ainsi les recombinaisons entre gènes portés par le même chromosome, (2) leur présence est indiquée par un marqueur phénotypique dominant qui est visible chez l'adulte (ou

¹ <http://flybase.org/>

la larve ou même la pupa) et (3) ils portent des mutations récessives qui sont létales ou réduisent la fécondité des individus homozygotes (Casso et al., 1999).

Si les chromosomes balanceurs ont permis la constitution de banques de mutants, c'est le système GAL4-UAS associé à un système de transgénèse efficace qui donne tout son sens à la drosophile comme modèle pour la physiologie. Chez la drosophile, la transgénèse est réalisée grâce à l'utilisation de transposons modifiés, des éléments P, qui sont injectés dans les mouches au stade embryonnaire. Alors que les premiers systèmes d'injection réalisaient des insertions au hasard, depuis plusieurs années maintenant, il est possible d'insérer les transgènes dans des sites relativement fixes, et donc mieux contrôler l'environnement génétique de ces gènes insérés (Venken and Bellen, 2007).

Le système UAS-GAL4 a été développé par Brand and Perrimon (1993). Ce système consiste à exprimer un gène rapporteur (GAL4), qui lui-même induit l'expression d'un second gène rapporteur. Cette induction est réalisée en plaçant devant le second gène rapporteur une séquence particulière, appelée « Upstream Activation Sequence » (UAS). Lorsque la protéine GAL4 se lie avec UAS, le gène placé en aval est activé et exprimé. Ce système est emprunté à la levure, *Saccharomyces cerevisiae*, qui utilise cette signalisation pour lui permettre de réagir rapidement à la présence de galactose dans l'environnement (Peng and Hopper, 2002). Chez *D. melanogaster*, il est donc possible de créer des souches de mouches qui portent différents éléments de cette signalisation, avec d'une part des souches exprimant GAL4 de manière « silencieuse » dans des cellules déterminées, et des souches portant dans leur génome une ou plusieurs constructions précédées du motif UAS. Lorsque l'on croise une souche GAL4 avec une souche UAS, les mouches issues de ce croisement vont alors exprimer le gène rapporteur dans les cellules où est exprimé GAL4 (Figure 9). Avec le temps, ce schéma a été adapté pour permettre une expression transitoire de GAL4, par exemple en utilisant une forme thermosensible de la protéine GAL80 (GAL80^{TS}) qui inhibe à température normale l'action de GAL4 mais pas à 30°C (Suster et al., 2004), et comme dans le système MARCM, en activant une flippase au cours du développement afin de marquer des cellules filles (Lee and Luo, 2001), ou enfin, en établissant de nouveaux systèmes d'expression binaire basés sur d'autres couples protéine / activateur comme LexA ou QF (Gohl et al., 2011).

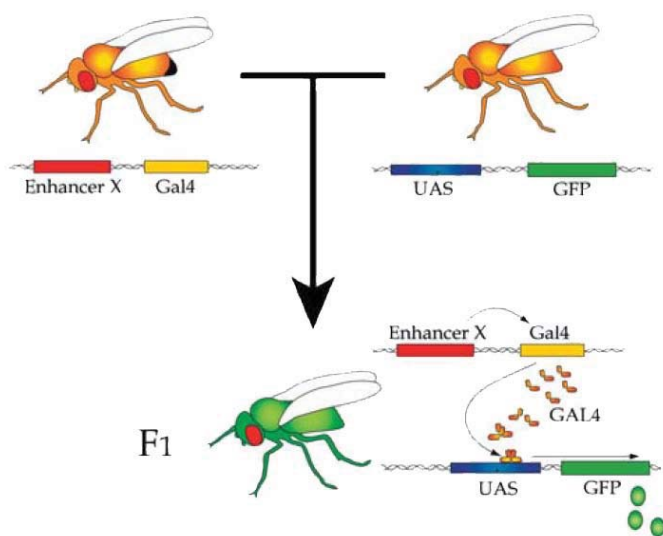


Figure 9 : Schéma du système UAS-GAL4 sur la Drosophile

Le principe de ce système est de croiser des mouches qui possèdent un gène rapporteur « neutre », i.e. la protéine GAL4 sous la dépendance d'un « enhancer » ou d'une région promotrice (notée « Gen1-Gal4 »), avec une souche de mouches qui portent un gène rapporteur placé en aval d'une séquence UAS (noté « UAS-Gen_rapp »). Les individus issus du croisement des deux parents (notés « Gen1 > Gen_rapp ») expriment Gen_rapp sous la dépendance de Gen1.
Figure extraite de [Ishimoto and Tanimura \(2004\)](#)

Dans notre travail, nous avons principalement utilisé deux constructions UAS, une toxine diphtérique modifiée (DTi) qui provoque la mort des cellules exprimant ce gène ([Bellen et al., 1992](#); [Han et al., 2000](#)), et la toxine pertussis (PTX) qui bloque l'activation des protéines G_o chez la drosophile ([Hopkins et al., 1988](#); [Fitch et al., 1993](#)). En utilisant le promoteur de Gr66a, qui est exprimé dans des cellules sensibles à des composés amers ([Thorne et al., 2004](#)), il est possible soit de supprimer ces cellules sensorielles (avec DTi), soit de perturber les événements transductionnels impliquant la protéine G_o . Etant donné que l'effet de ces toxines dépend de l'intensité de leur expression qui est elle-même liée à l'intensité de l'expression du gène rapporteur Gal4, il n'est pas certain que ces toxines aient l'effet désiré. Il faut donc à chaque fois comparer la réponse des neurones qui nous intéressent chez le mutant (par exemple *Gr66a > DTi*) avec celle des parents (*Gr66a-Gal4* ou *UAS-DTi*).

Cette revue rapide de la littérature concernant les relations plantes insectes et du système gustatif des insectes suggère que ce système sensoriel est utilisé comme un système prédictif de la qualité des aliments. Il est donc important de mieux le connaître afin de pouvoir mieux comprendre et prévoir le comportement alimentaire des insectes en présence de leur plante hôte. A cet égard, la drosophile représente un modèle expérimental de choix car c'est un insecte modèle qui permet de nombreuses manipulations génétiques qui ne sont pas possibles sur les insectes ravageurs eux-mêmes.

5. Principales méthodes expérimentales utilisées dans ce travail

a) Matériel biologique

Les mouches utilisées sont issues d'un élevage en laboratoire sur milieu artificiel (contient de l'agar, de levure, de gaude, de nipagine et d'alcool) maintenues à 25°C. L'origine des souches utilisées est indiquée en annexe. Afin d'obtenir des mouches dont les cellules sensibles à l'amer sont supprimées, nous avons utilisé une approche génétique consistant à croiser des mouches UAS-DTi et Gr66a-Gal4, qui nous permettent d'obtenir des mouches en F1 dont les cellules L2 (qui expriment Gr66a) sont tuées. Dans tout ce qui suit, nous avons utilisé des mouches d'environ 4-5 jours.

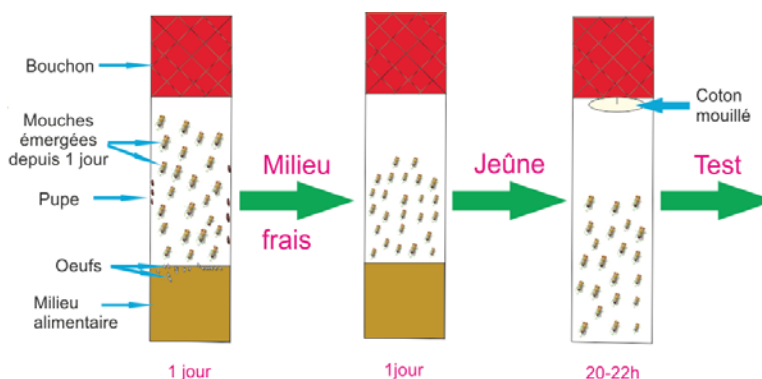


Figure 10 : Schéma présentant les différentes étapes de préparation des mouches pour les tests comportementaux (MultiCAFE et PER).

b) Test alimentaire à choix multiples (MultiCAFE)

Le protocole utilisé pour nos observations reprend la procédure utilisée au laboratoire (Sellier et al., 2011). Brièvement, les mouches émergées sont placées sur un milieu alimentaire frais pendant 1 jour et ensuite placées dans des tubes contenant un papier filtre ou un coton mouillé pendant 20-22 h pour les faire jeûner (Figure 10). Ces mouches sont ensuite transférées par groupes de 20 dans des boîtes rectangulaires en polystyrène crystal transparent (95 x 76 x 15 mm, Caubère) dans lesquelles ont été disposés 5 à 6 capillaires de 5 µl (Hirschman Laborgewäre, VWR), remplis d'une solution test. Ces boîtes sont alors scannées par groupes de 6 et placées à l'obscurité pendant 2 h. A la fin de l'expérience, ces boîtes sont à nouveau scannées et les images analysées sous ImageJ (Abramoff et al., 2004), afin de mesurer le niveau de liquide disparu, dans les boîtes d'expérience et dans des boîtes témoin (sans mouches) afin d'éliminer la disparition de liquide due à l'évaporation.

c) Test d'extension du proboscis

Nous avons aussi utilisé une autre approche comportementale qui est appelée extension du proboscis (PER) (Figure 11). Les insectes sont anesthésiés en les plaçant dans un tube de verre plongé dans de la glace pilée (2-3 minutes au plus) et ensuite disposés sur du patafix (UHU stick), et immobilisés sur le dos grâce à une bandelette de scotch placée sur le thorax, en veillant à laisser libre l'extrémité des pattes prothoraciques afin de pouvoir les stimuler avec un cure-dent trempé dans la solution test.

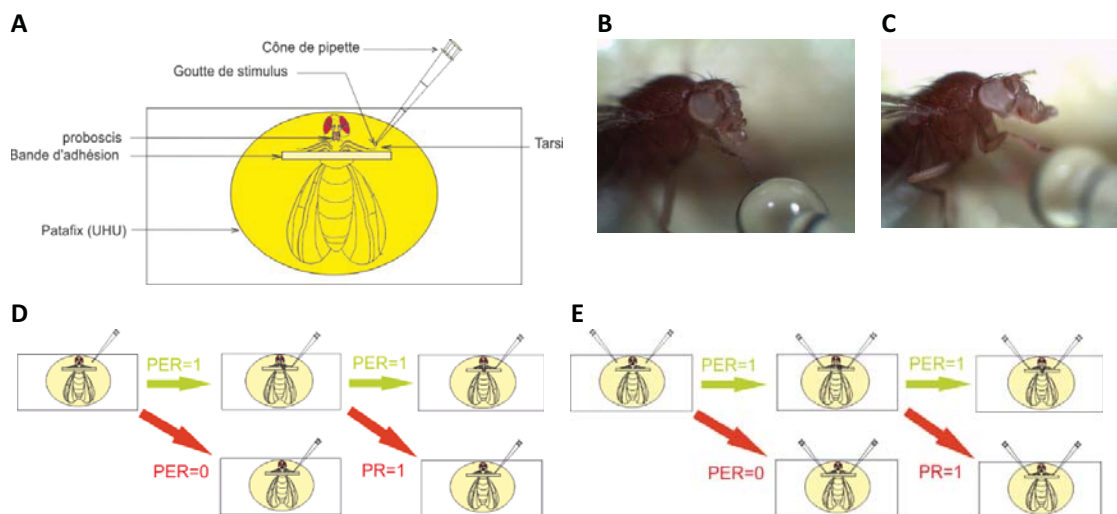


Figure 11 : Schéma des procédures utilisées pour mesurer l'extension du proboscis (PER).

(A) les mouches sont immobilisées sur une lame de microscope et on stimule le tarse avec un cure-dents en bois, trempé dans la solution de test ; (B) Réponse de la mouche à une solution amère (le proboscis est activement rétracté) ; (C) Extension du proboscis en réponse à une solution sucrée ; (D) Stimulation des tarsi avec un mélange de sucre et de substance amère ; (E) stimulation dissociée (sucre d'un côté et amer de l'autre). Les réponses observées sont l'extension du proboscis (PER) et la rétraction du proboscis (PR) si l'insecte rétracte le proboscis et le maintient rétracté.

Lorsque l'on touche une patte ou le proboscis avec une solution sucrée (soit au bout d'une pipette, soit en trempant un cure-dents en bois dans la solution test), les insectes étendent le proboscis (Wang et al., 2004; French et al., 2015b). Le pourcentage d'insectes qui étendent le proboscis est dose-dépendant. Si l'on mélange un composé amer au sucre, l'extension du proboscis peut être inhibée en fonction de la concentration du composé amer. On peut également présenter les stimuli de manière dissociée, en stimulant un tarse avec la solution sucrée et l'autre avec le stimulus amer (Dethier, 1976; Lacaille et al., 2007). Afin d'éviter les faux positifs, avant chaque stimulation, l'insecte est stimulé avec de l'eau. La réponse comportementale observée est l'extension du proboscis (PER : proboscis étendu pendant 5 s) et la rétraction du proboscis (PR : proboscis rétracté (observé après une extension)).

d) Enregistrements électrophysiologiques

Les enregistrements réalisés sont obtenus dans ces expériences en couvrant l'extrémité d'une soie gustative avec un capillaire de verre contenant la solution stimulante et un électrolyte pendant au moins 2 s. Après avoir été brièvement anesthésié en le plaçant sur de la glace pilée (pas plus de 5 min), l'insecte est disposé sur du Patafix (UHU stick) sur le flanc et immobilisé avec 2-3 bandelettes de papier adhésif (Scotch crystal) de 0.2-05 mm x 1 cm, afin d'exposer les sensilles du proboscis (Figure 12c). L'électrode de référence est un simple fil d'argent placé contre l'insecte et mis en contact avec celui-ci à l'aide d'une goutte de gel d'électrocardiographie. Les enregistrements électrophysiologiques sont sauvegardés et analysés à l'aide d'un logiciel développé au laboratoire, dbWave.

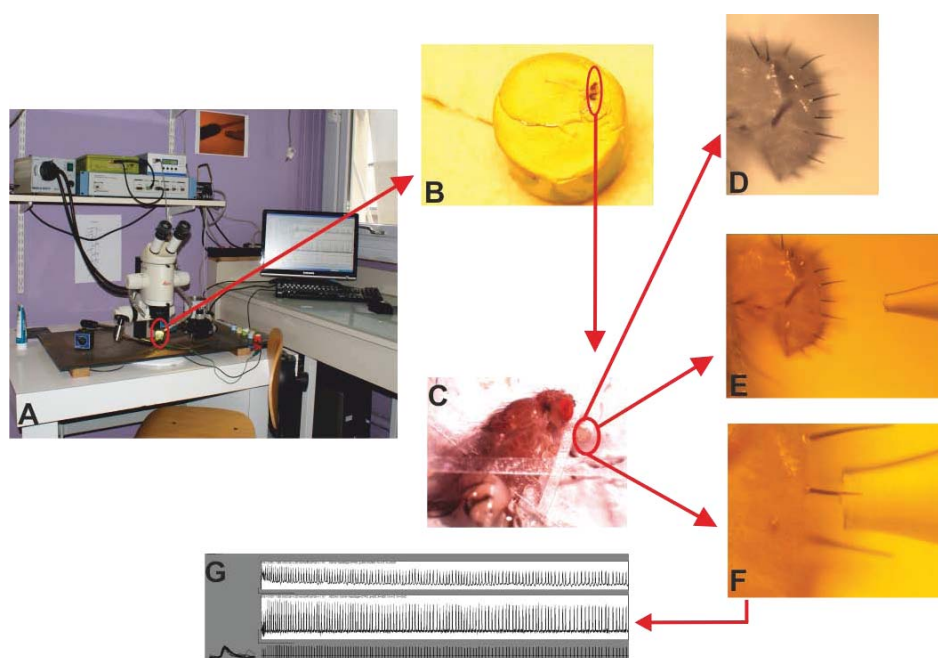


Figure 12 : Photographie du dispositif d'électrophysiologie et des étapes de la stimulation d'une sensille gustative du proboscis.

(A): Poste d'électrophysiologie de gustation comprenant une loupe, un éclairage par lumière froide, un système d'amplification et d'acquisition de données et la préparation placée sur un plot magnétique, posé sur un bras articulé permettant de changer son orientation. **(B)** Vue du plot magnétique, sur lequel est immobilisée une mouche posée sur du Patafix. **(C)** Vue d'ensemble de la mouche immobilisée par des bandelettes de papier adhésif transparent. **(D,E,F)** Procédure de stimulation d'une sensille : le proboscis est maintenu immobile et la position des sensilles est identifiée. Un capillaire de verre contenant la solution stimulante et relié à un amplificateur par un fil d'argent est approché sous contrôle visuel grâce à un micromanipulateur afin de coiffer la sensille choisie. **(G)** Vue de l'écran d'analyse des enregistrements. La trace du haut représente le signal enregistré pendant 2 s. Afin de détecter les potentiels d'action, ce signal est filtré et on ajuste un seuil de détection afin d'extraire de cet enregistrement tous les événements correspondant au dépassement de ce seuil. Les événements extraits sont des blocs de 60 points associés à un temps et à un numéro de classe. Si le seuil est correct, ces événements correspondent à des potentiels d'action qui sont affichés dans la ligne du dessous sous deux formes : des potentiels superposés (afin d'estimer combien de classes de potentiels sont présentes) dans le cartouche de gauche, et sous forme de bâton afin de visualiser leur distribution temporelle.

D. Objectifs généraux de la thèse

Le point de départ de notre travail a été une publication concernant le système gustatif de la drosophile, publié par [Mitri et al. en 2009](#) dans PLoS Biology. Dans cet article, les auteurs montraient que les drosophiles sont capables de détecter et d'éviter un acide aminé non protéique produit par des légumineuses, la L-canavanine. La détection de ce pseudo acide aminé impliquait un nouveau type de récepteur gustatif, DmXR, qui était exprimé dans des neurones des sensilles gustatives sur les pièces buccales, les pattes et probablement dans les neurones de l'organe gustatif épipharyngien ([Mitri et al., 2009](#)). Ce récepteur était complètement atypique en ce sens qu'il appartient à la famille des récepteurs au glutamate et n'avait aucun lien ni avec la famille des Grs, ni avec les IRs. Ce récepteur est un récepteur métabotropique, ce qui avait été montré dans un travail antérieur par les mêmes auteurs, à la fois par sa proximité structurelle avec les récepteurs du glutamate et en l'exprimant dans un système hétérologue, des cellules HEK ([Mitri et al., 2004](#)). En outre, un récepteur similaire à DmXR avait été trouvé chez d'autres insectes comme l'abeille ou le moustique ([Mitri et al., 2004](#)), ce qui suggérait une pression de sélection forte pour en maintenir la fonction dans des espèces très éloignées. Dans leur travail de [2009](#), les auteurs montraient que DmXR était exprimé dans des cellules exprimant Gr66a (donc des cellules a priori sensibles à l'amer), et que les mouches dont le récepteur était muté n'étaient plus capables d'éviter de consommer la L-canavanine.

Nous avons donc envisagé un travail commun afin de montrer que la détection de la L-canavanine impliquait bien des cellules gustatives détectant des composés amers comme la strychnine ou la caféine, et agissait sur le comportement alimentaire de manière similaire. Au cours de notre travail, un laboratoire concurrent ayant publié que la détection de la L-canavanine impliquait des récepteurs gustatifs classiques, Gr8a, Gr98a et Gr66a ([Lee et al., 2012](#); [Shim et al., 2015](#)), nous avons abandonné nos travaux sur DmXR, en complétant toutefois notre analyse de la détection des substances amères par deux aspects, d'une part en étudiant l'impact de la L-canavanine sur la détection des sucres, d'autre part en participant à la démonstration que la détection de L-canavanine implique l'intervention de protéines G, contrairement à la caféine. Cet ensemble de travaux fait l'objet d'un premier chapitre.

Nous avons alors réorienté notre travail afin de développer une question parallèle à celle qui était alors traitée au laboratoire, à savoir l'influence des mélanges sur la détection des substances simples. Notre laboratoire avait démontré que chez la drosophile, la présence de substances amères comme la strychnine ou la quinine avait un effet inhibiteur sur la détection des sucres ([Meunier et al., 2003b](#); [Sellier et al., 2011](#)). Cette observation était examinée par Alice French (French, 2014). J'ai

d'abord participé à la question posée par Alice en effectuant des observations électrophysiologiques et comportementales, sur l'inhibition du goût sucré par des substances amères. J'ai ensuite abordé une question miroir, qui est de savoir si les sucres inhibent la détection des substances amères. Cette question a été jusqu'à présent très peu étudiée et à notre connaissance, une seule publication a abordé explicitement cette question ([Cocco and Glendinning, 2012](#)). Ces travaux sur la détection de mélanges de substances amères et sucrées sont rassemblés dans un second chapitre.

Enfin, j'ai participé à plusieurs collaborations initiées à la demande de laboratoires extérieurs. Le premier travail concerne l'impact d'analogues d'un neuropeptide sur la détection de substances gustatives. Ce travail avait pour point de départ la synthèse de neuropeptides modifiés, analogues à la kinine, par le professeur Nachman et l'analyse de leurs effets sur le comportement alimentaire d'*Aedes aegypti* par le Professeur Patricia Pietrantonio (Texas A&M, USA). Nous avons donc évalué la réponse électrophysiologique de sensilles gustatives de moustiques au laboratoire. Par ailleurs, nous avons testé l'impact de ces analogues sur les réponses gustatives de drosophiles, à la fois en électrophysiologie et en comportement. Le second travail concerne la détection de L-arabinose et de D-arabinose. Le professeur Kausik Si (University of Kansas Medical Center, USA) avait développé un travail montrant que les drosophiles mémorisent mieux la présence de L-arabinose que celle de D-arabinose, bien que cette seconde molécules soit préférée (travail en cours de publication). J'ai contribué à ce travail en effectuant des enregistrements électrophysiologiques sur la détection de ces substances, et en encadrant une étudiante qui a réalisé des tests comportementaux de consommation. Ces travaux sont rassemblés dans un troisième chapitre.

Enfin, ce manuscrit se termine par une discussion et une conclusion générale. Les originaux des articles publiés ou soumis sont placés en annexe de ce document.

II. Détection de la L-canavanine et de composés amers

A. Introduction

De nombreuses plantes produisent des acides aminés « non protéiques » qui sont soit des intermédiaires du métabolisme primaire, soit des substances secondaires impliquées dans leur défense (Huang et al., 2011). Au moins 200 molécules de ce type ont été identifiées dans les plantes, pour la plupart dans des graines de légumineuses (Fowden, 1981). La plus étudiée est la L-canavanine, qui est présente dans les graines de plus de 350 espèces de Légumineuses (Bell, 2003 ; Rodgers and Shiozawa, 2008). Cette molécule est toxique pour un grand nombre d'organismes comme les bactéries, champignons, levures, algues, plantes, insectes et mammifères (Huang et al., 2011). Elle est confondue par le métabolisme avec la L-arginine car les protéines intégrant la L-canavanine n'ont pas la même structure tri-dimensionnelle et ne sont pas fonctionnelles (Rosenthal and Dahlman, 1986). Certains insectes comme *Caryedes brasiliensis* ou *Heliiothis virescens* sont capables de détoxifier cette molécule et peuvent même l'utiliser comme source d'azote (Rosenthal, 1983, 2001).

La détection gustative de la L-canavanine est moins bien connue. Il est vraisemblable que beaucoup d'insectes phytophages soient capables de détecter ce composé, soit directement, soit indirectement, sans que cela ait été analysé de manière spécifique. Par exemple, les expériences de toxicité effectuées sur des larves de *Manduca sexta* indiquent que les chenilles consomment moins de nourriture en présence de L-canavanine (Dahlman, 1977), ce qui pourrait être dû non seulement à la toxicité de ce composé, mais aussi à son goût amer. Dans un travail réalisé sur des larves de 5 espèces de Lépidoptères, Simmonds et Bradley mentionnent que la L-canavanine stimule les sensilles D des sensilles styloconiques médiales de ces larves (Simmonds et al., 1990). La L-canavanine a été décrite également comme capable d'inhiber le goût du saccharose chez *Schistocerca americana* (Chapman et al., 1991).

En 2009, l'équipe de Yves Grau avait montré qu'un autre insecte, la Drosophile, est capable d'éviter la L-canavanine qui possède un goût aversif (Mitri et al., 2009). Dans ce travail, les auteurs montraient non seulement que les drosophiles adultes évitent de consommer la L-canavanine de manière dose-dépendante, mais aussi que cette aversion disparaissait quand un gène récepteur était affecté, DmXR. Ce récepteur est exprimé dans les neurones gustatifs, particulièrement dans les dendrites. L'expression de ce récepteur dans les neurones sensibles à l'amer de drosophiles mutantes pour DmXR restaure la capacité des mouches à détecter la L-canavanine. La nouveauté de cette observation tient au fait que DmXR s'écarte des récepteurs gustatifs classiques (GRs, IRs).

II.- Détection de la L-canavanine

DmXR appartient à une famille de récepteurs au glutamate, qui sont des récepteurs avec un motif extracellulaire de type « venus flytrap » couplés à des protéines G (Figure 13).

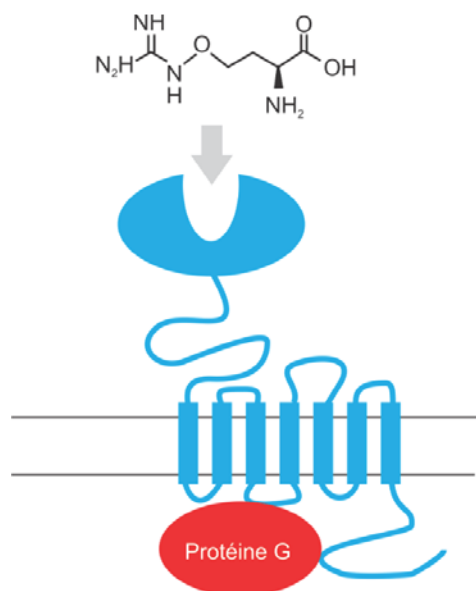


Figure 13 : Représentation hypothétique du couplage de DmXR avec la L-canavanine

Les récepteurs de type Venus flytrap ont une partie extracellulaire comportant un site de liaison avec un ligand et 7 domaines transmembranaires. La partie C-terminale est intracellulaire et interagit avec des protéines G. DmXR fonctionnerait comme sur ce schéma et serait exprimé dans des neurones gustatifs de drosophiles (Mitri et al., 2009). Schéma modifié d'après Pin et al. (2003).

Ce travail s'appuyait sur des observations antérieures démontrant qu'il existe chez la drosophile, mais aussi chez *Aedes gambiae* et *Apis mellifera*, un récepteur de type XR sensible à un ligand contenant un groupe amine, différent du glutamate et présent dans des extraits de cerveau d'insectes (Mitri et al., 2004). Ce récepteur métabotrope appartient à la famille des récepteurs au glutamate, habituellement exprimés dans les synapses, par exemple musculaires (chez les insectes). Lorsque DmXR est transfecté dans ces cellules HEK, ces cellules répondent à la L-canavanine et sont inhibées par la *N*-methyl-L-arginine, connue pour inhiber la synthèse d'oxyde nitrique à partir de L-arginine (Mitri et al., 2009). Ils montraient également, en utilisant différentes mutations induites par des éléments transposables insérés dans un gène appelé *mangetout* (*mtt*) ou dans sa région promotrice (Figure 14), que DmXR était impliqué dans la détection gustative de la L-canavanine. Dans flybase, le gène *mangetout*² est répertorié comme ayant 5 transcrits annotés et 5 polypeptides ; 15 allèles ont été répertoriés. Ce gène est appelé *DmXR* dans notre travail et dans les publications de Mitri et al. Il a d'autres synonymes comme CG8692, CG18447, CG30361, DmGluBR, DmGluRB, GluRB, Glu-RB, DmXR et mXr.

² <http://flybase.org/reports/FBgn0050361.html>

II.- Détection de la L-canavanine

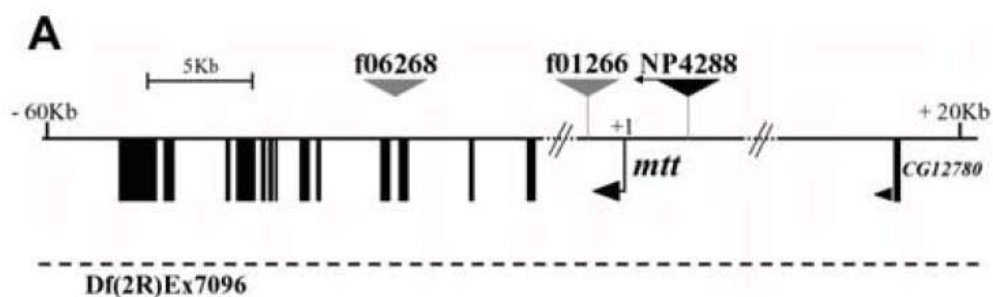


Figure 14 : Constructions génétiques utilisées pour analyser la fonction gustative de *mtt* (Mitri et al., 2009)

Le gène *mtt* est un gène identifié par Mitri et al. (2004) comme étant un récepteur métabotrope. Flybase répertorie 5 transcrits différents (*mtt*-RC, RD, FR, RG et RE). Les lignées utilisées sont :

- *f06268* qui contient un transposon piggyBac à 35 pb du 3^{ème} exon de *mtt*
- *f01266* qui contient un transposon piggyBac à 1.7 kb du début de *mtt*
- *NP4288-GAL4* qui est une lignée *Gal4*, inséré à 3.1 kb avant *mtt*

En outre, ce travail utilisait une lignée d'excision, *Df(2R)Ex7096*, qui comprend *mtt* et touche également les gènes adjacents (*CG8697* et *CR2397*).

Comme les expériences publiées ne comportaient pas d'observations électrophysiologiques et compte tenu de la nouveauté de ces recherches, nous avons d'abord cherché à préciser quels types de cellules gustatives répondaient à L-canavanine et avec quelle cinétique temporelle. En effet, DmXR étant couplé à une cascade de protéines G, on pouvait s'attendre à des réponses qui se développent plus lentement que dans le cas de récepteurs ionotropiques. Une telle cinétique aurait pu expliquer en particulier, pourquoi la L-canavanine n'affectait pas les réponses d'extension du proboscis mais activait une réponse de rétraction postérieure à l'extension (Mitri et al., 2009). Nous avons ensuite participé à un travail mené principalement à Montpellier, consistant à démontrer que la détection de L-canavanine impliquait une protéine G, contrairement à la détection de la caféine.

B. *Gr66a* est impliqué dans la détection de la L-canavanine

1. Introduction

Nous avons cherché à préciser quelles cellules gustatives répondent à la L-canavanine et avec quelle cinétique. Afin de disposer d'une référence positive, nous avons comparé les réponses électrophysiologiques ou comportementales à la L-canavanine aux réponses obtenues avec de la strychnine et la caféine. Ces substances sont connues pour avoir un effet aversif sur les mouches, à la fois en comportement car elles inhibent de manière dose-dépendante l'alimentation (Meunier et al., 2003b; Sellier et al., 2011), et elles sont détectées par des cellules répondant aux substances amères (Meunier et al., 2003b). La détection de la caféine implique notamment le récepteur gustatif *Gr66a*, *Gr93a* (Lee et al., 2009; Moon et al., 2009), tandis que la strychnine impliquerait également *Gr47a* (Lee et al., 2015). De manière générale, les cellules répondant aux substances amères (sur le

proboscis) expriment *Gr66a*, tandis que celles qui répondent aux sucres expriment *Gr5a* et *Gr64f* (Scott et al., 2001; Thorne et al., 2004; Wang et al., 2004; Amrein and Thorne, 2005; Jiao et al., 2007; Montell, 2009).

Afin de démontrer le rôle des cellules gustatives exprimant *Gr66a*, nous avons comparé les réponses électrophysiologiques et comportementales de mouches chez lesquelles ces cellules étaient supprimées ou chez lesquelles la transmission synaptique était inhibée. Nous avons ensuite étudié le rôle spécifique de *Gr66a*, *Gr33a* et *DmXR* sur la détection de ces substances amères en testant des mutants pour ces récepteurs.

2. Les cellules exprimant *Gr66a* sont nécessaires à la détection de L-canavanine

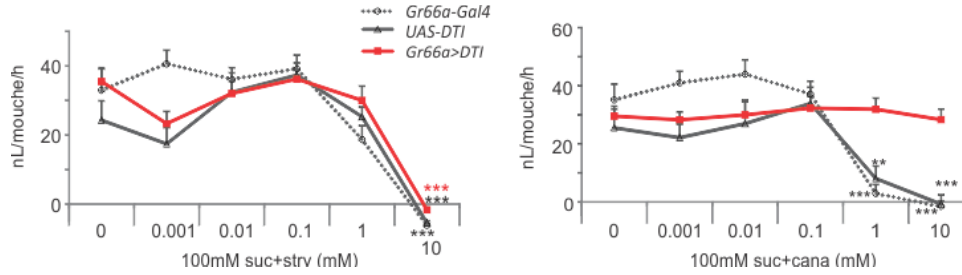
En utilisant des souches GAL4 construites avec une séquence promotrice de *Gr5a* ou de *Gr66a*, nous avons exprimé une toxine diphtérique dans ces cellules afin de les supprimer (UAS-DTi). En comportement, nous avons testé l'effet de la L-canavanine sur la consommation de sucre (saccharose 100 mM) additionné de L-canavanine ou de strychnine, en utilisant un protocole d'observation qui permet de mesurer la quantité de liquide consommé à partir de capillaires multiples (Sellier et al., 2011). En électrophysiologie, sur le proboscis, nous nous sommes focalisé sur les cellules « L2 » (Meunier et al., 2003b) qui ont été identifiées comme des cellules répondant aux substances amères, plus particulièrement sur les sensilles S6 qui répondent à la L-canavanine.

Les mouches contrôle (*i.e.* UAS-DTi et *Gr66a-Gal4*) ainsi que les mouches dont la cellule L2 est supprimée (*Gr66a > DTi*), évitent de consommer les solutions contenant de la strychnine en fonction de la dose (Figure 15 : Consommation de saccharose en présence de L-canavanine et de strychnine chez des mouches dont les cellules sensibles à l'amer (exprimant *Gr66a*) sont inactivées par une toxine (DTI ou TeTxLC). Par contre, alors que les mouches contrôle peuvent détecter la présence de L-canavanine, les individus dont les cellules L2 sont supprimées ne détectent pas la L-canavanine en mélange avec du saccharose (Figure 15A). Pour confirmer ces observations, nous avons utilisé la toxine du tétanos (tetanus toxin light chain ou TeTxLC). Cette toxine ne tue pas les cellules comme DTI mais empêche la transmission synaptique (Sweeney et al., 1995). Les résultats obtenus sont qualitativement similaires (Figure 15-B).

Nous avons testé si les mouches dont les cellules qui expriment *Gr66a* sont supprimées par DTi sont toujours capables de répondre à la canavanine ainsi qu'à la strychnine (et à la caféine). Nous avons effectué des enregistrements sur la sensille s6. Sur la souche mutante *Gr66a > DTi*, on observe une disparition des réponses à la L-canavanine (40 mM), à la strychnine (10mM) et à la caféine (10mM) (Figure 16), contrairement aux les souches parentales qui répondent aux trois substances.

II.- Détection de la L-canavanine

A : *Gr66a* > *DTi*



B : *Gr66a* > *TeTxLC*

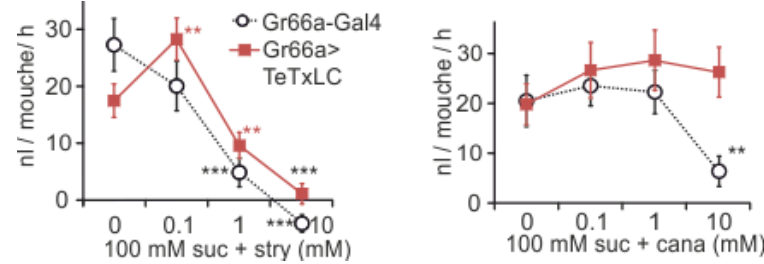


Figure 15 : Consommation de saccharose en présence de L-canavanine et de strychnine chez des mouches dont les cellules sensibles à l'amer (exprimant *Gr66a*) sont inactivées par une toxine (*DTi* ou *TeTxLC*)

(A) Effets de l'inactivation des cellules *Gr66a* par la toxine diphtérique. Consommation moyenne de groupes de 20 mouches ($n=8-10$ répétitions) dont les cellules exprimant *Gr66a* sont supprimées par *DTi* (en rouge) ou des mouches contrôle (en gris), de strychnine (*stry* = 0, 0.001, 0.01, 0.1, 1, 10 mM) ou en présence de L-canavanine (*l-cana* = 0, 0.001, 0.01, 0.1, 1, 10 mM) en mélange avec du saccharose (100 mM) et de l'erioglaurine B pour colorer le liquide. (B) Effets de l'inactivation des synapses des cellules *Gr66a* par *TeTxLC*.

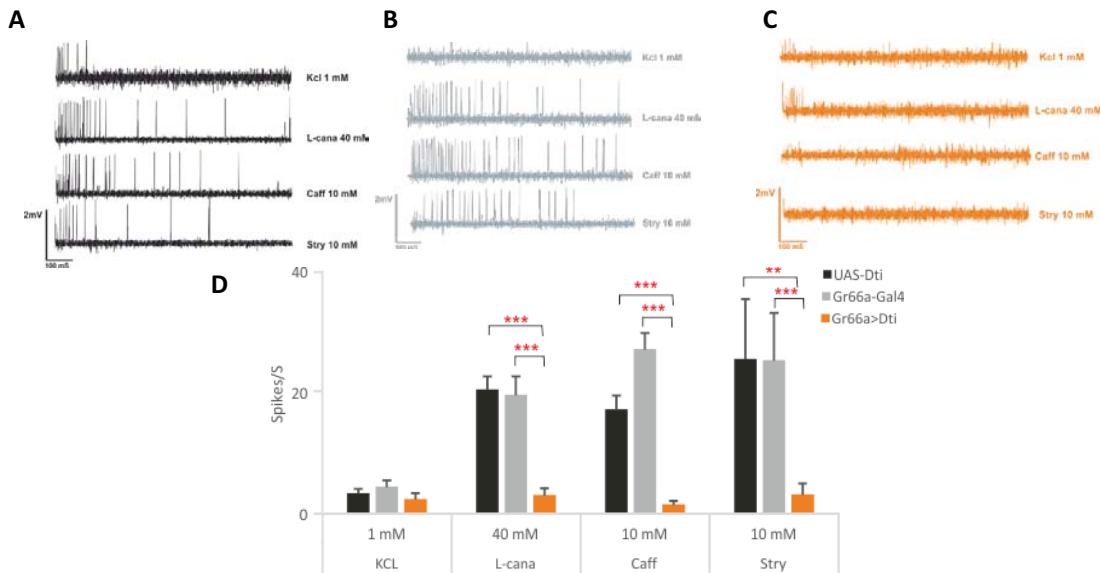


Figure 16 : Réponses des sensilles *s6* à la canavanine, à la strychnine et à la caféine chez des mouches témoin ou dont la cellule exprimant *Gr66a* est inactivée

(A-C) Exemples d'enregistrements obtenus sur les souches parentales A : *UAS-DTi* (noir), B : *Gr66a-Gal4* (gris), C : *Gr66a > DTi* (orange). (D) Activité observée en réponse au KCl 1 mM, à la L-canavanine (40 mM), à la caféine (10 mM) et à la strychnine (10 mM). Les réponses observées chez les parents sont absentes lorsque les cellules exprimant *Gr66a* sont supprimées. Tests statistiques : Mann-Whitney U Test pour comparer la souche *Gr66a-DTi* avec chacun de deux parents *UAS-DTi* ($p < 0.001, 0.001, 0.01$ pour *l-cana*, *caff* et *stry* respectivement) et *Gr66a-Gal4* ($p < 0.001$). $n=6-11$.

En résumé, nos résultats indiquent que les cellules exprimant GR66a sont bien responsables de la détection de L-canavanine. Leur suppression ou l'inactivation de leurs synapses empêchent les mouches de détecter ce composé en comportement et en électrophysiologie. Ce n'est pas le cas de la strychnine pour laquelle on continue à observer une aversion comportementale à cette substance dans dans le protocole comportemental.

3. *Gr66a* (mais pas *Gr33a* ni *DmXR*) est essentiel à la perception de L-canavanine

Dans le paragraphe précédent, nous avons vu que les cellules exprimant *Gr66a* étaient nécessaire à la détection de L-canavanine. Ces cellules expriment d'autres récepteurs (Weiss et al., 2011), et en particulier *Gr33a* (Moon et al., 2009) et *DmXR* (Mitri et al., 2009). Afin de vérifier que ces récepteurs jouent un rôle (ou pas) dans la réponse à la L-canavanine et à d'autres composés amers, nous avons testé la réponse comportementale et électrophysiologique de mutants de ces récepteurs.

Les mutants de *Gr66a* et *Gr33a* proviennent du laboratoire de Montell. *Gr66a^{ex83}* a été obtenu par une excision de la région du gène (Moon et al., 2006) ainsi que de deux gènes adjacents (Figure 17). Cette excision induit une déficience dans la détection de la caféine (mais pas de la quinine).

Les mutations induites dans *Gr33a* ont été obtenues par recombinaisons homologues en incorporant le gène *mini-white* et *Gal4* dans le gène (Moon et al., 2009). Nous avons testé un seul de ces mutants, *Gr33a¹* (Figure 18). Le mutant *DmXR* provient du travail de Mitri et al. (2009). Dans ce qui suit, nous avons testé *Df(2R)EX7096* (Figure 14).

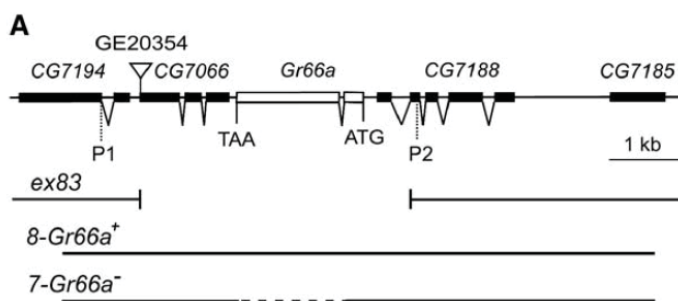


Figure 17 : Inactivation du gène *Gr66a* par excision (Moon et al., 2006)

Carte physique de la région génomique de *Gr66a*. *Gr66a* est inséré sur le 3^{ème} chromosome. L'excision *ex83* concerne 3 gènes, *CG7066*, *Gr66a* et *CG7188*. Ces deux gènes n'ont apparemment pas d'effets sur les phénotypes mesurés (comportement alimentaire, électrophysiologie gustative).

II.- Détection de la L-canavanine

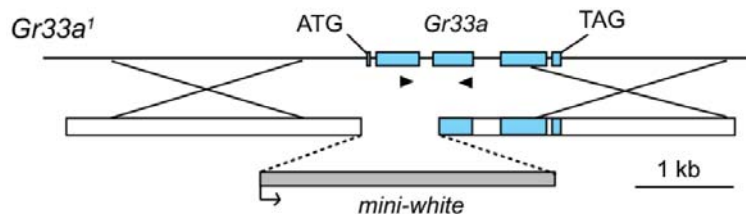


Figure 18 : Inactivation du gène *Gr33a* par recombinaison homologue (Moon et al., 2009)

Structure de l'allèle *Gr33a*¹ obtenu par recombinaison homologue. Cet allèle présente des déficiences sur les réponses électrophysiologiques à des substances amères (caféine, quinine, berbérine, denatonium, lobeline, papavérine, strychnine).

Nous avons d'abord réalisé des observations comportementales avec un test multiCAFE (Figure 19). Les mouches témoin (*w*¹¹¹⁸) évitent de consommer la solution sucrée contenant de la caféine 30 mM ou de la L-canavanine 30 mM. Les mutants *Gr66a*^{ex83} sont incapables de détecter la caféine ou la L-canavanine aux concentrations testées. Le niveau de consommation des mouches *Df(2R)EX7096* est trop faible pour que les résultats soient significatifs.

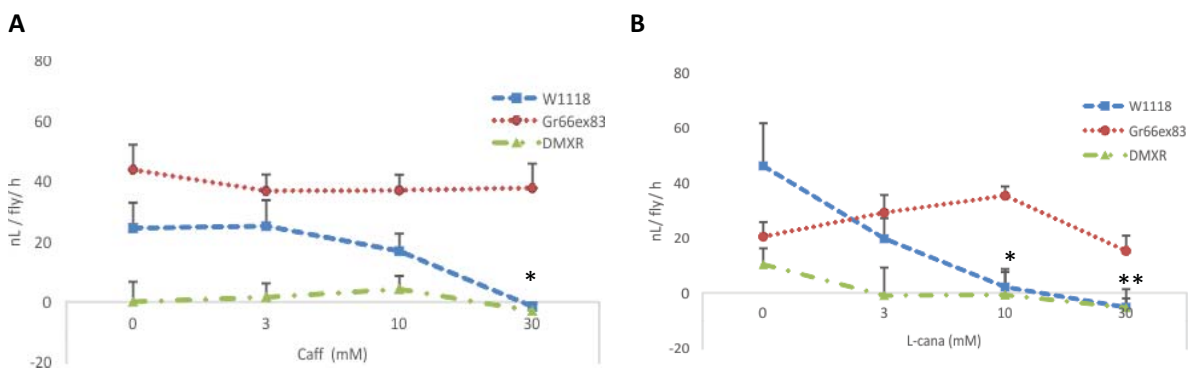


Figure 19 : Consommation de saccharose en présence de caféine et L-canavanine chez des mutants *Gr66a* et *DmXR*.

Des groupes de 20 mouches ($n = 8-10$) ont été testés pour leur consommation de saccharose 100mM mélangée à une substance amère. Ces mouches étaient des adultes issus des souches *w*¹¹¹⁸ (contrôle), *Gr66a*^{ex83} (mutant *Gr66a*) et *Df(2R)EX7096* (mutant *DmXR*). (A) Consommation de saccharose 100 mM mélangée avec de la caféine (Caff) à 0, 3, 10 et 30 mM. Conformément à ce qui était attendu, la souche mutante pour *Gr66a* ne détecte pas la caféine alors que la souche contrôle évite la concentration la plus élevée. Le mutant *DmXR* consomme très peu. (B) Consommation de saccharose 100 mM en présence de L-canavanine (L-cana) à 0, 3, 10 et 30 mM. Comme pour la caféine, la mutation du gène *Gr66a* altère la détection de L-cana. Le mutant *DmXR* consomme très peu. Les astérisques sont placés sur les points (moyenne + SEM) qui sont significativement différents de la consommation de saccharose seul, selon le test Wilcoxon Matched Pairs. Pour la souche *w*¹¹¹⁸ p value= 0.028418 ; 0.005062 pour L-cana10-30 mM et $p=0.016605$ pour la caféine 10 mM.

II.- Détection de la L-canavanine

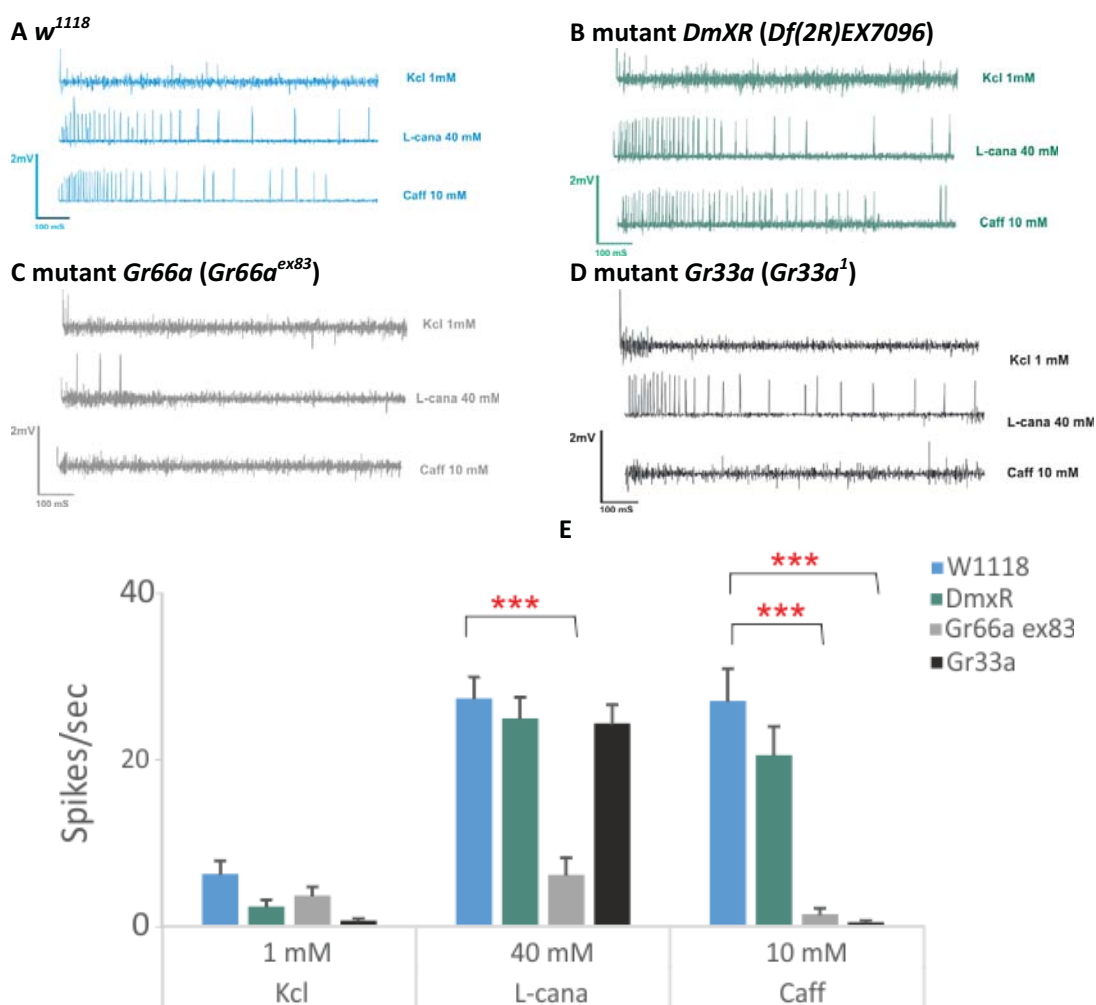


Figure 20 : Réponses gustatives de la sensille s6 à la L-canavanine et à la caféine chez des mutants *Gr66a*, *Gr33a* et *DmXR*

Les enregistrements effectués sur la sensille s6 ont été réalisés en tip-recording, en coiffant la sensille pendant 2 s avec une électrode de stimulation. Le nombre de potentiels d'action détectés pendant la 1^{ère} s est mesuré. (A-D) Exemples d'enregistrements obtenus sur les différentes souches. (E) Réponses obtenues sur les sensilles s6 (moyenne \pm SEM ; n=10 individus). Les astérisques correspondent à la comparaison entre le témoin et le mutant par un test de Mann-Whitney U (*w¹¹¹⁸* vs *Gr66a^{ex83}* l-cana : $p = 0.000049$, caff : $p=0.000052$; *w¹¹¹⁸* vs *Gr33a* caff : $p=0.000142$).

Nous avons complété ces observations comportementales par des observations électrophysiologiques. Pour ce faire, nous avons enregistré les réponses des sensilles s6 du proboscis à la canavanine et à la caféine, en testant les souches mutées pour *Gr66a* et *DmXR* ainsi qu'une souche mutée sur le gène *Gr33a*. Les enregistrements ont été réalisés en utilisant de la caféine 10 mM et de la canavanine 40 mM. Nos résultats montrent que les mouches qui sont mutées sur *Gr66a* ne répondent plus aux deux substances, tandis que celles qui sont mutées sur *Gr33a* continuent à répondre à la canavanine mais ne sont plus capables de détecter la caféine. Par contre, la mutation sur *DmXR* ne produit aucun effet apparent (Figure 20).

Ces résultats indiquent que *DmXR* et *Gr33a* n'interviennent pas dans la détection de la L-canavanine et confirment que *Gr66a* joue un rôle important, au moins comme co-récepteur nécessaire dans les sensilles s6.

4. DmXR n'affecte pas la détection de L-canavanine

Nous avons voulu confirmer ou bien infirmer l'absence de rôle de DmXR dans la perception de la L-canavanine en testant la descendance de croisements entre deux mutants de ce récepteur, *mtt^{f06268}* et *Df(2R)Ex7096* (avec ou sans GFP). Les observations ont été réalisées en électrophysiologie seulement, sur les sensilles s5 et s6 du proboscis. Un de deux parents a été testé ainsi que les deux descendants *mtt^{f06268}; mtt^{f06268} / Df(2R)Exel7096* et *mtt^{f06268} / Df(2R)Exel7096/Cyo* actin GFP). Toutes ces mouches ont émis des potentiels d'action en réponse à la L-canavanine (40mM) et la caféine (10mM) (Figure 21).

Enfin, nous avons réalisé une expression ectopique de DmXR dans des neurones sensibles au sucre, en utilisant une souche GAL4 incluant un promoteur de *Gr5a*. La construction génétique UAS-DmXR a été réalisée par Yves Grau. Si DmXR est un récepteur pour la L-canavanine, alors les cellules répondant au sucre et exprimant *Gr5a*, devraient répondre à la L-canavanine. Les résultats obtenus sur cette souche mutante *Gr5a > DmXR* sont regroupés en (Figure 22). Nous n'observons pas de différence entre les réponses de cette souche mutante et les souches parentales pour la L-canavanine, ni pour les autres substances testées (saccharose, caféine et mélanges binaires).

L'ensemble de ces résultats confirme le rôle de *Gr66a* dans la détection de la L-canavanine mais pas celui de DmXR. Nos observations sont compatibles avec les deux publications du groupe de Montell qui ont démontré que *Gr66a*, *Gr8a* et *Gr98b* sont nécessaires pour détecter la L-canavanine (Lee et al., 2012; Shim et al., 2015).

II.- Détection de la L-canavanine

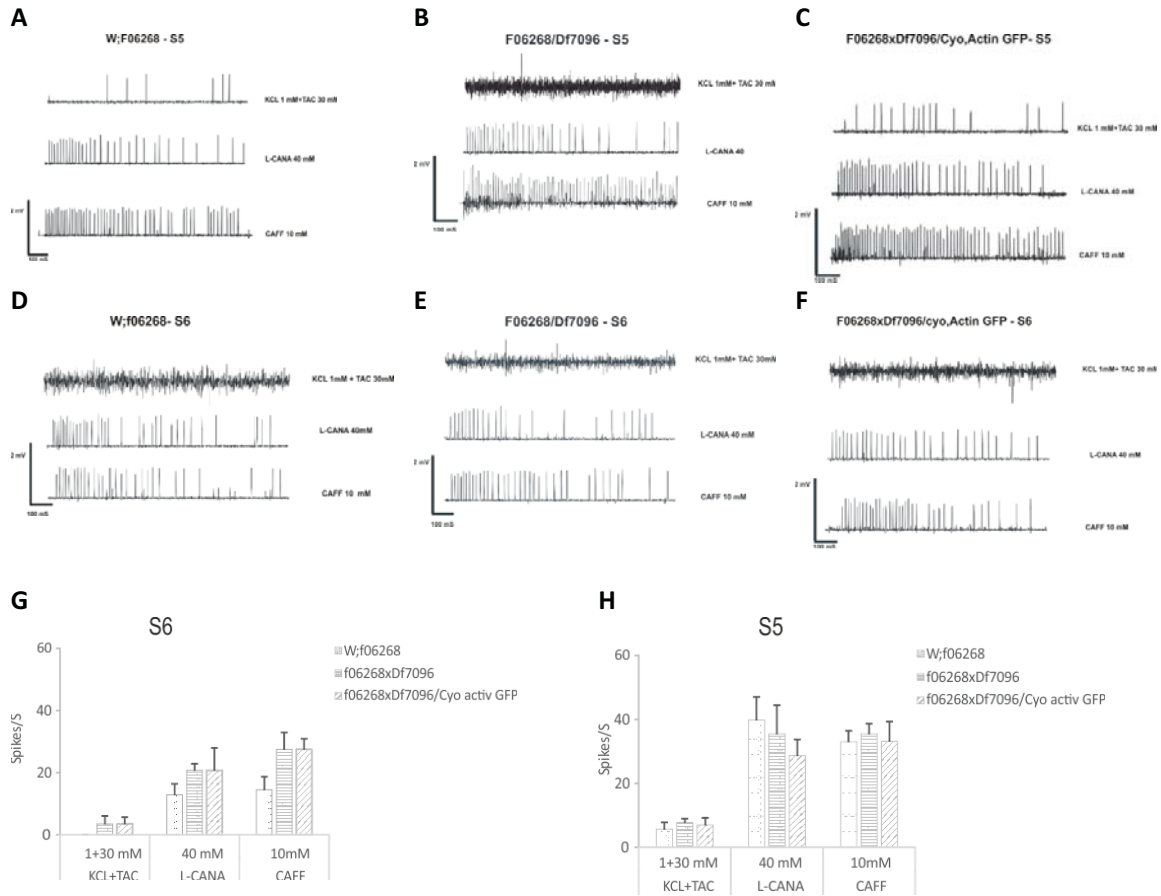


Figure 21 : Réponses électrophysiologiques de S5 et S6 chez des mutants de DmXR à la canavanine et à la caféine

(A-F) Exemples de réponses électrophysiologiques. (G-H) Moyenne (\pm SEM) des réponses à la L-canavanine (L-CANA) et à la caféine (CAFF). La L-canavanine est détectée par les souches parentales et les descendants malgré l'absence de DmXR sur les deux sensilles S5 et S6. Le test statistique (Mann-Whitney U Test) ne montre aucune différence significative entre la souche parentale (w;f06268) et les mutants. n=6.

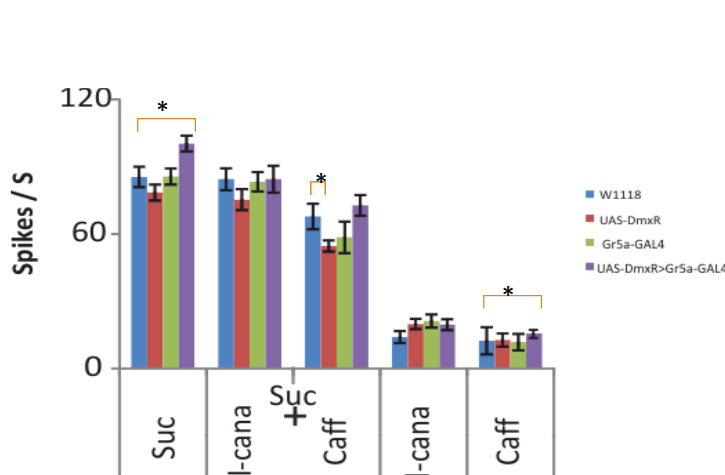


Figure 22 : Réponses de S6 à L-canavanine dans des mouches Gr5a > DmXR

Dans cette expérience, nous avons exprimé DmXR dans des cellules répondant aux sucres (Gr5a > DmXR) et mesuré la réponse de sensilles L6 (sans cellule sensible à l'amer). Nous n'observons pas de différences statistiques entre les réponses du mutant et les souches parentales (test Mann-Whitney U) pour le saccharose 100 mM, la L-canavanine 30 mM (l-cana), la caféine 10 mM (Caff) ou le mélange sucre + l-cana / sucre + caff. n= 10-11.

C. $G\alpha_o$ est essentielle à la détection de la L-canavanine [article 1]

1. Introduction

Alors que les récepteurs olfactifs et gustatifs des insectes ont longtemps été considérés comme des récepteurs couplés à des protéines G comme ceux des Vertébrés (Clyne et al., 2000), différentes observations ont démontré qu'ils sont insérés dans les membranes de manière inversée (Benton et al., 2006; Lundin et al., 2007; Zhang et al., 2011). Cette observation semble exclure que ces récepteurs puissent être couplés à des protéines G, puisque leurs sites de liaison classiques aux protéines G sont extracellulaires. Actuellement, les récepteurs olfactifs sont considérés comme des pores ioniques hétéromultimériques qui n'auraient pas besoin de protéines G pour changer la perméabilité des membranes des neurones. Certains de ces récepteurs pourraient former des canaux fonctionnels homodimériques (ou multimériques) comme le récepteur au saccharose chez la mouche à viande, *Boettcherisca peregrina* (Murakami and Kijima, 2000), ou bien *BmGr9* qui est sensible au fructose chez *Bombyx mori* quand il est exprimé dans un système d'expression hétérologues (Sato et al., 2011). Différentes expériences consistant à exprimer ces récepteurs olfactifs ou gustatifs dans des cellules en culture ou des œufs de xénope (Sato et al., 2008; Sato et al., 2011) montrent que certains de ces récepteurs peuvent fonctionner comme un canal cationique. L'équipe de Montell a même montré que lorsque *Gr66a*, *Gr8a* et *Gr98b* sont co-exprimés dans des cellules de drosophile S2, ils forment un canal cationique non sélectif qui est activé par la L-canavanine (Shim et al., 2015).

Cependant, la plupart de ces données sont obtenues en exprimant des récepteurs dans des cellules qui sont équipées d'un ensemble de voies de transduction fonctionnelles, qui pourraient jouer un rôle dans les réponses observées. En ce qui concerne l'olfaction, des travaux suggèrent que les protéines G jouent un rôle dans la transduction et dans la modulation de l'intensité des réponses (Wicher et al., 2008; Sargsyan et al., 2011; Getahun et al., 2013; Raja et al., 2014; Miazzi et al., 2016). Cependant, les effets observés en l'absence de protéines G ou de messagers secondaires, concernent plutôt une modulation de l'intensité de la réponse, en particulier la phase tardive. L'effet des protéines G semble plus marqué en gustation, où des observations sur la drosophile et d'autres espèces d'insectes suggèrent que des protéines G modulent la détection de ligands comme des sucres (Ueno et al., 2006; Kain et al., 2010; Bredendiek et al., 2011) ou certaines substances amères (Ouyang et al., 2009). Dans la plupart de ces travaux, l'identité des protéines G impliquées n'est pas connue avec certitude car la démonstration utilise une approche pharmacologique, par exemple un inhibiteur compétitif de l'activation des protéines G comme GDP β S (Koganezawa and Shimada, 1997) ou un inhibiteur de la voie IP₃ comme U73122 (Ouyang et al., 2009). Enfin, l'équipe de J. Carlson (Yale) a montré que si les récepteurs olfactifs n'ont pas besoin de protéines G pour produire une réponse

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olfactive, les récepteurs gustatifs impliqués dans la détection du CO₂, GR21a et GR63a, ont besoin de G α_q et Gy30A (Yao and Carlson, 2010).

Ces protéines G sont des hétérotrimères constitués de trois sous unités α (alpha) β (bêta) et γ (gamma). Chez les mammifères, ces protéines sont codées par 35 gènes, dont 16 pour la sous-unité G α , 5 pour G β et 14 pour G γ (Milligan and Kostenis, 2006). La sous-unité α comprend quatre familles en prenant en considération les similarités de séquence: G α_i , G α_s , G α_q , et G $\alpha_{12/13}$ (Simon et al., 1991; Cabrera-Vera et al., 2003; Milligan and Kostenis, 2006). Les G α_i sont connues généralement comme inhibiteurs de l'adénylate cyclase ; au contraire, les G α_s sont associées à l'activation de l'adénylate cyclase, les G α_q sont liées à la stimulation de la phospholipase C tandis que les G $\alpha_{12/13}$ sont probablement impliquées dans l'activation de la voie de la signalisation Rho (Cabrera-Vera et al., 2003; Riobo and Manning, 2005). Dans l'état inactif, la sous-unité G α forme un complexe avec les deux autres sous-unités β et γ sous la forme (G $\alpha\beta\gamma$), G α étant également liée à une molécule de GDP (guanosine diphosphate). Le modèle classique considère que la liaison du récepteur à un agoniste induit un changement de conformation du récepteur, qui lui permet de s'associer avec la protéine G. Cette liaison libère le GDP qui est remplacé par un GTP (guanosine triphosphate), présent à des concentrations intracellulaires plus élevées. Cette liaison avec le GTP dissocie la sous-unité G α du complexe G $\beta\gamma$, ce qui lui permet d'activer ou d'inhiber des autres cibles ou bien des messagers secondaires, comme des enzymes ou des canaux ioniques. Le GTP lié à l'unité G α est ensuite hydrolysé par une GTP_{ase}, permettant à la G α de se réassocier avec le complexe G $\beta\gamma$ (Figure 23) (Milligan and Kostenis, 2006).

Tableau 2 : Sous-unités G α et effecteurs associés chez les vertébrés (Cabrera-Vera et al., 2003)

Family	Subtype	Effector
G _s	G $\alpha_{s(S)}$ ^a	↑ AC
	G $\alpha_{s(L)}$ ^a	↑ GTPase of tubulin ^b
		↑ src ^b
	G α_{olf}	↑ AC
G _i	G α_{i1}	↓ AC
	G α_{i2}	Rap 1 GAP
	G α_{i3}	GRIN 1 and 2
	G α_{oA} ^a	↑ GTPase of tubulin ^b
	G α_{oB} ^a	↑ src ^b
	G α_z	Ca ²⁺ and K ⁺ channels ^b
	G α_{t1}	↑ cGMP-PDE
	G α_{t2} G α_g	Unknown
G _q	G α_q	↑ PLC β s
	G α_{11}	↑ Bruton's tyrosine kinase (G α_q)
	G α_{14}	
	G α_{15} or 16	
G ₁₂	G α_{12}	↑ NHE-1 ^b
		↑ PLD ^b
	G α_{13}	↑ p115RhoGEF ↑ iNOS ^b

*PDE, phosphodiesterase E;
iNOS, oxyde nitrique synthase induite
NHE, échangeur Na/H
PLD, phospholipase D
GEF, facteur d'échange de guanosine*

II.- Détection de la L-canavanine

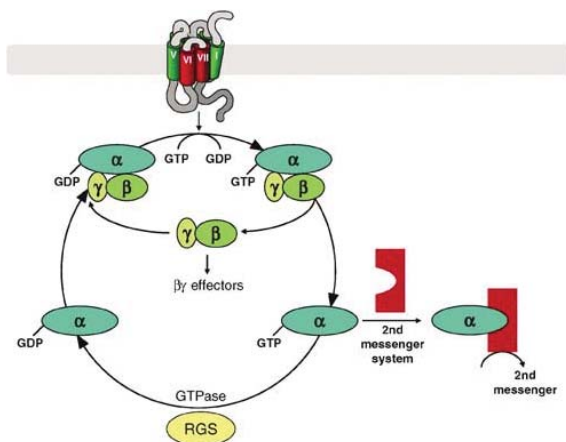


Figure 23 : Récepteurs couplés aux protéines G et protéines G hétérotrimériques.

Schéma des différentes étapes de l'activation de la protéine G. (RGS= régulateurs de la signalisation des protéines G) d'après [Milligan and Kostenis \(2006\)](#).

Tableau 3 : Gènes codant pour des protéines G chez la drosophile

	Symbole	Synonymes	CG	Source
G alpha	cta	concertina , conc	CG17678	http://flybase.org/reports/FBgg0000463.html
	Gαf	Gα73B, Galpha73B, Gfα, Gf	CG12232	
	Gαi	Gα _i , G-ialpha65A, G-ia65A, Giα, Gi	CG10060	
	Gαo	G-οα47A, G-oalpha47A, Go, Goα, Gα _o	CG2204	
	Gα		CG40005	
	Gαs	Gα _s , Gs, Gsα, dgs, Gsα60A	CG12232	
	Gαq	dga, Gα49B, Galpha49B, Gqα, Gα _q	CG17759 CG17760 CG30054	
G	Gβ5		CG10763	http://flybase.org/reports/FBgg0000464.html
beta	Gβ13F	Gb13F	CG10545	
	Gβ76C	Gβ Gβe, Gβ76C	CG8770	
G	Gγ30a	D-Gγ1, Gγ, l(2)k08017, bro4	CG3694	http://flybase.org/reports/FBgg0000465.html
gamma	Gγ1	Gγ, Gγe, Gye, lincRNA.S701	CG8261	

La drosophile possède également une famille de gènes codant pour des protéines G (Tableau 3) : 9 gènes codent pour une protéine Gα (6 sont exprimées dans les antennes : *concertina*, *Gα_q*, *Gα_s*, *Gα73B*, *Gα_o*, et *Gα_i* ([Yao and Carlson, 2010](#)), les deux autres étant CG40005, CG17760 et CG30054), 3 gènes codent pour une protéine Gβ (Gβ76C, Gβ5 et Gβ13F) et deux gènes pour Gγ (Gγ30a et Gγ1) ([Hanlon and Andrew, 2015](#)). A notre connaissance, aucun travail n'a répertorié les protéines G exprimées spécifiquement dans les neurones gustatifs périphériques.

Afin d'inhiber sélectivement l'une des protéines G, nous avons exprimé le gène de la toxine pertussis (PTX) dans les cellules gustatives. Cette exotoxine est normalement produite par une

bactérie *Bordetella pertussis*, responsable de la coqueluche chez l'homme. La PTX est composée de 5 sous-unités. Chez les mammifères, la première sous-unité (S1) perturbe la signalisation transmembranaire par ADP-ribosylation des sous-unité $G\alpha_o$ et $G\alpha_i$ (Katada et al., 1983). Les cinq autres sous-unités (S4 comprend 2 formes), sont responsables de la liaison de la toxine à la surface cellulaire chez les eucaryotes (Stein et al., 1994). Chez la drosophile, cette toxine est plus sélective et se lie seulement à $G\alpha_o$ car $G\alpha_i$ ne possède pas de cystéine à laquelle cette toxine se lie normalement (Provost et al., 1988).

2. Influence de la protéine G sur les réponses électrophysiologiques et comportementales

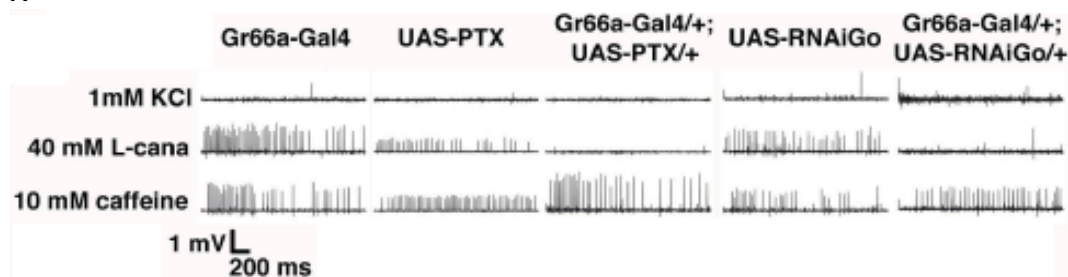
Le travail que nous avons réalisé sur ce sujet est un travail expérimental en complément d'une question abordée par Isabelle Devambez au cours de sa thèse (INSERM, Montpellier). Il s'agissait d'évaluer si la réponse à la L-canavanine ou à la caféine nécessitait la présence d'une protéine G. Ces deux substances sont détectées sur le proboscis par des cellules exprimant *Gr66a*. Nous avons réalisé les observations électrophysiologiques (tip recording) et comportementales (MultiCAFE) sur des mouches témoin (souches parentales) et exprimant une toxine de pertussis pour un article publié en 2013 (Devambez et al., 2013), article qui est joint en annexe.

Nous avons complété ces observations en testant la réponse à 3 autres substances, la théophylline (proche de la caféine), la strychnine et la berbérine (Figure 24C). Comme pour la caféine, nous n'avons pas observé de réduction de l'activité aussi bien chez des mutants *Gr66a* > PTX que chez des mutants *Gr66a* > RNAi/Go.

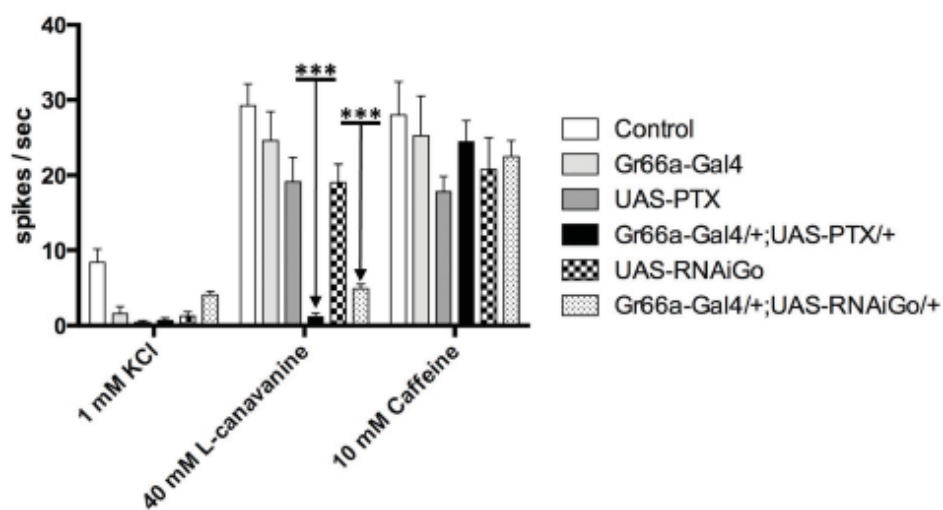
Il est intrigant de noter que la même cellule nerveuse utilise des voies de transduction différentes selon les ligands (caféine versus L-canavanine), ligands qui sont apparemment reconnus par des récepteurs de même type, c'est-à-dire des Grs. Il serait intéressant de tester si l'expression ectopique des récepteurs à la L-canavanine dans les neurones sensibles aux sucres comme l'ont réalisé Shim et al. (2015), implique la même protéine G, ou si cette relation est spécifique des neurones sensibles à l'amer.

II.- Détection de la L-canavanine

A



B



C

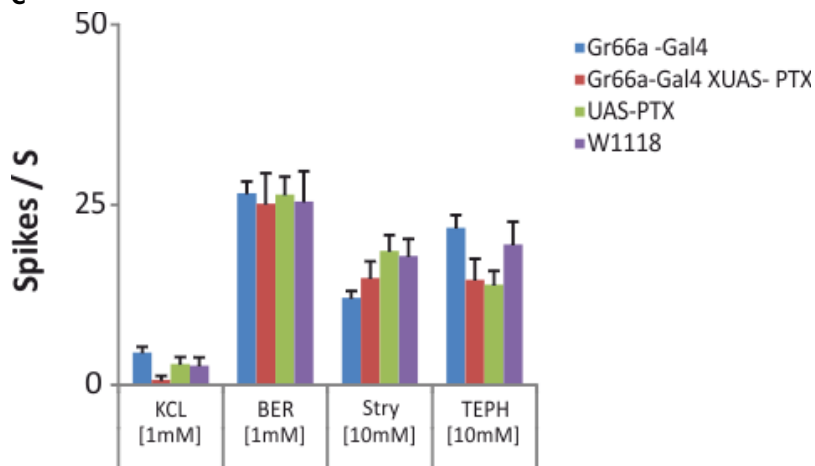


Figure 24 : La désactivation de $G\alpha_{o47A}$ par PTX supprime les réponses à la L-canavanine (mais pas à d'autres substances amères)

L'inhibition de $G\alpha_{o47A}$ par PTX conduit à une disparition de la détection de la L-canavanine (A-B) Réponses électrophysiologiques obtenues de sensilles S6 du proboscis. La réponse à la L-canavanine (40 mM) est inhibée par PTX. (C) Aucun effet n'est observé sur les réponses aux autres molécules (caféine, strychnine, théophylline et berbérine). Les astérisques indiquent à la différence significative test Mann-Whitney. $n = 7-10$

D. Discussion

Dans une première partie, nous avons montré que la détection de la L-canavanine impliquait une population spécifique de cellules gustatives. Ces cellules sont caractérisées par l'expression de GAL4 lorsque *Gal4* est inséré dans une construction comprenant une séquence promotrice du gène *Gr66a*. Ces cellules sont impliquées dans la détection des substances amères. Nous avons observé que des mouches chez lesquelles ces cellules sont supprimées ne détectent plus la L-canavanine à la fois dans un protocole de test comportemental alimentaire et en électrophysiologie. La suppression de ces cellules abolit la réponse électrophysiologique également à la strychnine et à la caféine, alors que pour la strychnine, son pouvoir aversif est maintenu dans les tests comportementaux.

Nous avons également cherché à confirmer le rôle de DmXR dans la détection de la L-canavanine en comparant la réponse des mutants DmXR disponibles à celle de mutants de gènes gustatifs impliqués dans la détection de substances amères, *Gr66a* et *Gr33a*. Nos observations électrophysiologiques et comportementales indiquent que *Gr66a* est directement impliqué dans la détection des substances amères et dans la détection de L-canavanine, alors que *Gr33a* ne participe pas à cette détection. Nous n'avons pas observé de différence notable dans la détection de L-canavanine lorsque DmXR est altéré.

Enfin, nous avons contribué à un travail visant à évaluer le rôle de protéines G dans la détection de L-canavanine (Devambez et al., 2013). L'ensemble de nos résultats indique que $G\alpha_{047A}$ est impliqué dans la détection de la L-canavanine mais que cette protéine G n'a aucun effet sur la détection des autres substances amères testées. Lorsque $G\alpha_{047A}$ est altérée, l'inhibition de la réponse à la L-canavanine qui est observée est drastique, bien plus que pour la détection des sucres (Ueno et al., 2006; Kain et al., 2010; Bredendiek et al., 2011). Ce résultat est tout à fait surprenant car les travaux de Montell concernant la détection de L-canavanine impliquent directement 3 récepteurs de la famille des Grs, *Gr66a*, *Gr8a* et *Gr98b* qui formeraient un canal cationique non sélectif activé par la L-canavanine (Shim et al., 2015).

Le travail que nous avons mené n'a pas confirmé les observations initiales de Mitri et al. (2009) qui proposaient que la L-canavanine est détectée grâce à un récepteur gustatif d'une nouvelle classe, *DmXR*. Nos résultats expérimentaux ont bien montré que *Gr66a* est nécessaire à cette détection mais réfuter le rôle de *DmXR* nous a pris beaucoup de temps. En outre, Lee et al. (2012) ont d'abord évalué de manière systématique les réponses à la L-canavanine de toute la population de sensilles de type s, ce qui leur a permis de concentrer leurs observations sur les trois sensilles les plus sensibles, à savoir S3, S5 et S10. En ce qui nous concerne, nous avons fait la plupart de nos observations sur la sensille S6 en faisant l'hypothèse que toutes les sensilles S avaient une sensibilité équivalente,

II.- Détection de la L-canavanine

puisqu'elles exprimaient DmXR (Mitri et al., 2009). Cette stratégie nous a conduit à obtenir des résultats beaucoup plus variables et peu clairs. Le second élément contradictoire avec les observations de Mitri et al. (2009) est que les souches mutantes testées par Montell et par nous-même, ne présentaient pas de différence marquée dans leur aversion pour la L-canavanine. Ce résultat étonnant est peut-être lié au rôle de DmXR dans la physiologie des insectes. En effet, ce récepteur est exprimé également dans le cerveau des insectes et semble très bien conservé (Mitri et al., 2004). Il pourrait être lié au rôle de l'arginine dans la physiologie des insectes, car l'on sait que cet acide aminé est un précurseur pour la production d'oxyde nitrique (NO) qui est un neurotransmetteur gazeux. Deux hypothèses peuvent alors être invoquées : (1) les souches mutantes auraient évolué et les insectes se sont adaptés à une mutation qui pourrait impliquer des déficiences multiples ; ces déficiences expliqueraient pourquoi les mutants testés consommaient beaucoup moins de liquide dans notre test MultiCAFE ; (2) le phénotype associé à la mutation de DmXR pourrait s'exprimer différemment en fonction des conditions d'élevage. Ces hypothèses sont testables expérimentalement. D'une part, il devrait être possible de contrôler le niveau d'expression de DmXR dans des animaux entiers, chez des mutants et des souches sauvages. D'autre part, il serait souhaitable de générer de nouveaux mutants affectant uniquement le gène *mtt*, par exemple en utilisant une approche CRISPR Cas9 (Yu et al., 2013). Les résultats surprenants obtenus en inhibant les protéines G α pour la détection de la L-canavanine mais pas pour d'autres substances amères montrent bien qu'il existe là une différence qui n'est pas explicable simplement.

Enfin, on pourrait invoquer une troisième hypothèse. Le récepteur DmXR joue probablement un rôle important dans la physiologie des insectes car il est exprimé non seulement dans les neurones gustatifs, mais aussi dans le cerveau, et surtout il semble très conservé évolutivement. Dans leur premier article, Mitri et al. (2004) montraient que ce récepteur était activé par une fraction d'extraits de cerveau. Il existe donc un ligand qui active ce récepteur de manière endogène et ce n'est pas la L-canavanine, qui est un composé provenant des plantes et qui est toxique après ingestion. Dans cette perspective, la L-canavanine est une substance toxique dont les animaux ont besoin de se protéger en évitant de l'ingérer. Il est donc concevable que le système gustatif des insectes ait évolué pour détecter de manière efficace ce composé, et que les deux systèmes de détection coexistent dans les mêmes neurones, mais ne jouent pas le même rôle.

III. Détection de L-canavanine et de composés amers dans un mélange binaire

A. Introduction

Dans ce qui précède, nous avons vu que la L-canavanine était détectée par les mêmes cellules que les cellules qui détectent l'amer, plus précisément, les cellules exprimant le récepteur *Gr66a* dans les sensilles du proboscis. Lorsque ces cellules sont supprimées (en exprimant une toxine de la diphtérie grâce à une construction UAS-DTI), nous avons observé que les réponses électrophysiologiques à la L-canavanine, à la strychnine et à la caféine, étaient complètement supprimées (Figure 16). Par contre, la consommation de solutions sucrées contenant l'une de ces substances amères est différente selon la molécule amère : alors que la L-canavanine n'a plus aucun effet, les mouches consomment beaucoup moins de solutions contenant de la strychnine (Figure 15). Cette différence pourrait s'expliquer par l'existence de cellules gustatives sensibles à la strychnine qui n'auraient pas été supprimées par la toxine. Une autre hypothèse serait que les deux tests biologiques évaluent des situations différentes : alors qu'en électrophysiologie nous avons testé des substances isolées, en comportement, il est nécessaire de présenter les produits amers en mélange avec des sucres. Ce qui est mesuré en comportement est en fait une baisse d'appétence aux sucres et non pas une réponse aux substances amères. Cette deuxième hypothèse rejoint des observations antérieures, réalisées dans notre laboratoire, qui montraient que l'exposition à une substance amère comme la quinine inhibait les réponses ultérieures au sucre pendant quelques minutes (Meunier et al., 2003b), et que la quinine mélangée à du sucre inhibait la réponse électrophysiologique à ce sucre (Sellier et al., 2011).

Des interactions entre composés sapides ont été décrites chez les vertébrés (Breslin, 1996) et chez les insectes (Glendinning, 2008). Cependant, les mécanismes qui président à ces interactions demeurent encore mal connus. Dans le domaine de l'olfaction, de nombreux auteurs ont noté également que les réponses comportementales sont souvent différentes si les composés sont présentés isolés ou en mélange, même pour des composés phéromonaux qui sont censés être très sélectifs (Party et al., 2013; Saraiva et al., 2016). Là encore, une multiplicité de phénomènes a été invoquée, comme des interactions au niveau du système nerveux central (Deisig et al., 2001), des interactions entre neurones adjacents à la périphérie (Su et al., 2012), des interactions au niveau des récepteurs protéiques (Oka et al., 2004) voire même des interactions entre les molécules elles-mêmes (Syed and Leal, 2008).

Au laboratoire, nous avons entrepris d'explorer les interactions possibles entre molécules « amères » et molécules « sucrées » chez la drosophile adulte. Cette interaction concerne à la fois les effets des substances amères sur la détection des sucres et les effets des sucres sur la détection de

composés amers. Les interactions amer → sucre ont fait l'objet de la thèse de Alice French (French, 2014). J'ai participé à l'analyse des effets des substances amères sur la détection des sucres en réalisant des observations électrophysiologiques et comportementales (MultiCAFE et PER) (French et al., 2015b). Mes observations sont décrites dans un premier sous-chapitre. En ce qui concerne les interactions sucre → amer, j'ai conduit une expérimentation visant à évaluer si les sucres interféraient avec la détection de substances amères. Ce travail fait l'objet d'un second sous-chapitre.

B. Interactions amer → sucre [article 2]

1. Introduction

Dans la première partie de ma thèse, nous avons remarqué que l'ablation des cellules exprimant Gr66a supprimait la détection de L-canavanine mais pas celle de strychnine, lorsque l'on mesurait la quantité de liquide sucré consommé par des mouches pendant 2 h (Figure 15). Cette différence pouvait être liée soit à l'existence d'une autre voie de détection gustative pour la strychnine qui ne serait pas affectée par l'ablation des cellules exprimant *Gr66a*, soit à un effet post-ingestif similaire à ce qui avait été observé pour l'abeille (Wright et al., 2010) et qui se manifeste pour la strychnine et pas pour la L-canavanine. A cet effet, j'ai réalisé des tests comportementaux d'extension du proboscis et des observations électrophysiologiques, afin de comparer l'effet de ces deux composés amers sur la détection de saccharose, sur un intervalle de temps beaucoup plus court afin d'écartier les effets post-ingestifs. Ce travail a été intégré dans une publication du laboratoire insérée en publications (French et al., 2015b). L'hypothèse principalement testée ici est que la strychnine inhibe directement les cellules répondant aux sucres, même en l'absence de cellules sensibles à l'amer, tandis que la L-canavanine n'a pas d'effet sur la réponse des cellules sensibles au saccharose.

2. Réponses comportementales PER/PR

Dans une première approche, nous avons noté la réponse d'extension du proboscis de mouches. Alors que classiquement, on note seulement l'extension du proboscis, nous avons également noté la rétraction du proboscis si l'insecte l'avait étendu auparavant (Figure 25). En effet, une rétraction retardée du proboscis avait été remarquée par Mitrì et al. (2009). Cette réponse retardée à la L-canavanine avait été considérée par les auteurs comme liée à un développement tardif de la réponse en liaison avec une voie de transduction métabotrope.

III.- Mélanges binaires sucré-amer

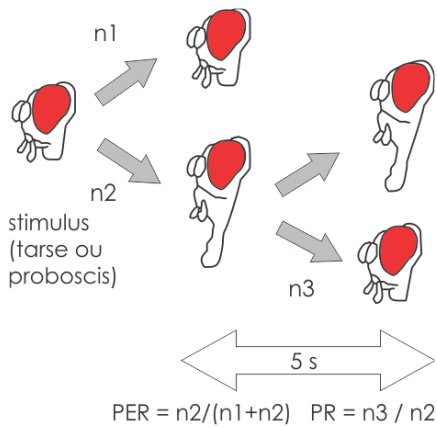


Figure 25 : PER / PR

Lorsque l'on stimule une mouche avec une solution sucrée, deux réactions peuvent être observées. La première est une extension du proboscis ou PER. Si l'on teste N individus, et que n2 répondent, $PER = n2/N$. Le deuxième comportement est une rétraction prolongée du proboscis consécutive à une extension ou PR, dans un intervalle de temps de 5 s après la stimulation. Si le nombre de mouches qui rétracte le proboscis est n3, $PR = n3/n2$.

Afin d'étudier l'indépendance des deux voies de détection (sucré / amer), nous avons stimulé les tarsi des mouches avec une solution sucrée en mélange avec une substance amère, ou bien en présentant le stimulus sucré et le stimulus amer sur deux pattes différentes (Figure 26 A). Si la détection des deux stimuli est réalisée par deux voies nerveuses indépendantes, on doit s'attendre à une réponse identique dans les deux cas.

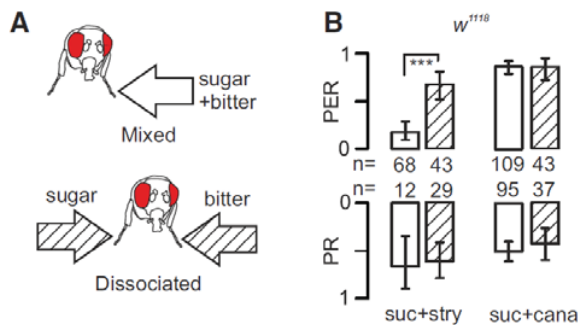


Figure 26 : PER / PR différent pour la strychnine et la L-canavanine selon le mode de stimulation (French et al., 2015b)

(A) Les tarsi peuvent être stimulés de deux manières : soit avec un mélange sucre + amer, soit en appliquant simultanément un stimulus sucré d'un côté et un stimulus amer de l'autre. (B) Sur des mouches w^{1118} , lorsque la strychnine est appliquée en mélange (barres blanches), elle réduit le PER plus que lorsque les stimuli sont séparés (barres hachurées). Par contre, le retrait du proboscis PR est similaire dans les deux conditions. Pour la L-canavanine, le PER et le PR ne sont pas affectés.

Une première expérience a été menée sur des mouches de souche w^{1118} (Figure 26B). Alors que les deux modes de présentation n'ont aucun effet sur la réponse PER à la L-canavanine, l'inhibition du PER est très marquée pour la strychnine en mélange mais pas lorsque la présentation est indépendante. Par contre, la réponse PR n'est pas affectée par le mode de présentation.

Nous avons ensuite effectué la même expérience sur des mouches $Gr66a>DTi$ (Figure 27 A-B). Nous retrouvons le résultat précédent concernant l'inhibition de la réponse PER à la strychnine en mélange mais pas lorsque les stimuli sont séparés, mais nous n'observons pas de différence significative de cette inhibition du PER chez les mouches $Gr66a > DTi$. Par contre, nous observons une

réduction significative du PR dans les deux types de présentation chez les mouches *Gr66a* > *DTi*. Comme *Gr33a* est considéré comme un corécepteur de *Gr66a* (Lee et al., 2009), nous avons testé des mouches *Gr33a* > *DTi* (Figure 27 C-D). Chez les mouches mutantes, nous retrouvons bien une inhibition du PR mais les effets sur le PER sont moins marqués.

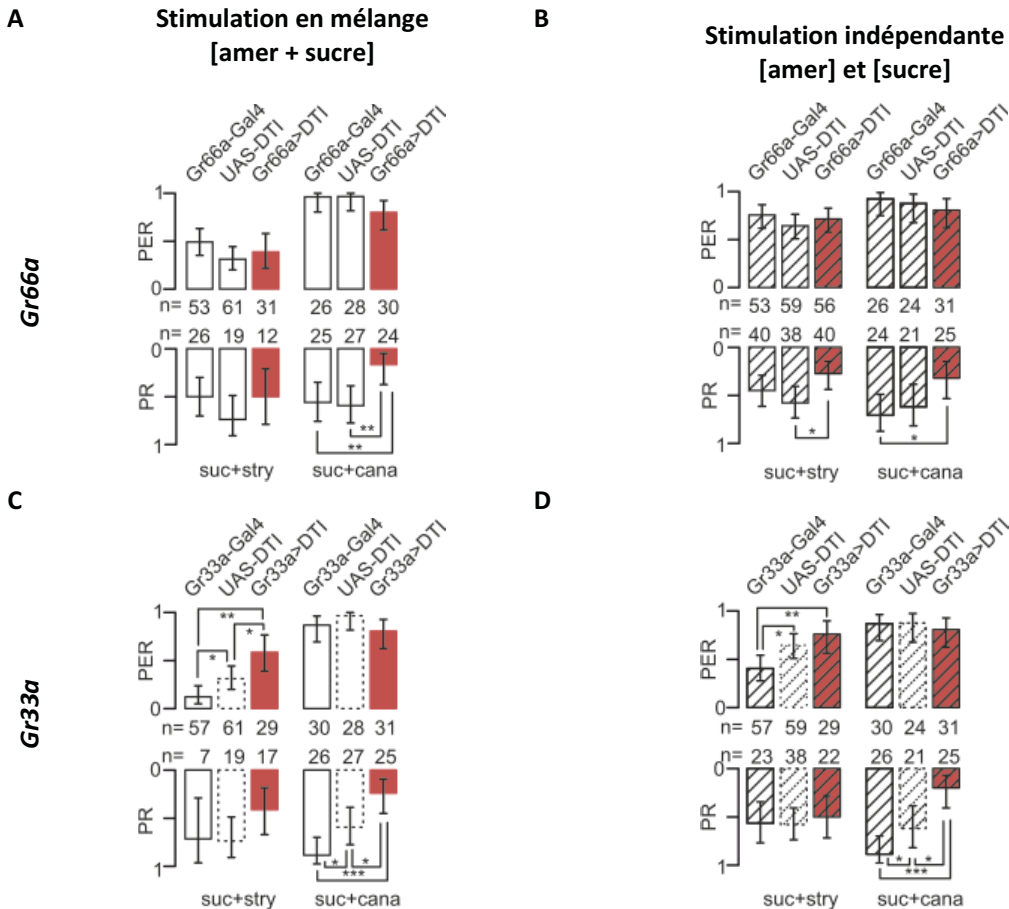


Figure 27 : PER/PR chez des mouches *Gr66a* > *DTi* et *Gr33a* > *DTi* (French et al., 2015b).

Nous avons mesuré la réponse PER et PR à du sucre (100mM), de la strychnine (10mM) ou de la L-canavanine (40 mM) et leurs mélanges respectifs sur des mouches dont les cellules exprimant *Gr66a* (A, B) ou *Gr33a* (C, D) ont été supprimées (mutant = barres rouges, souches parentales : barres blanches) à l'aide d'une toxine diphtérique. (A) (C) : stimulation avec un mélange. (B) (D) stimulation indépendante.

Ces résultats montrent qu'il existe une différence essentielle entre l'effet de la strychnine présentée en mélange ou séparément. Ces observations sont compatibles avec nos expériences effectuées en mesurant la consommation en MultiCAFE. Même si la strychnine exerce un effet post-ingestion, les différences observées en PER montrent que les mouches sont capables de détecter la strychnine en mélange avec du sucre de manière immédiate mais que cette détection est retardée

lorsque les stimuli sont présentés séparément. Cet effet sur le PER n'est pas du tout observé dans le cas de la L-canavanine. Si l'on supprime les cellules sensibles à l'amer, exprimant *Gr66a* ou *Gr33a*, nous n'observons pas d'altération majeure du PER. Par contre, la réponse PR est très fortement réduite. De ces expériences, on peut conclure que la réponse PR est fortement dépendante de la stimulation de cellules répondant à l'amer. Par contre, la réponse d'extension initiale du proboscis serait principalement sous la dépendance de l'activation de cellules répondant aux sucres. Le fait qu'une réponse inhibitrice apparaisse avec retard ou non pourrait dépendre de l'intensité relative des stimuli sucré et amer, ou bien, comme le suggéraient Mitri et al. (2009), d'une réponse nerveuse qui s'établit plus lentement dans le cas de la L-canavanine.

3. Réponses électrophysiologiques de sensilles du proboscis

Afin de lever l'ambiguïté qui subsiste concernant la réponse de rétraction du proboscis (PR) observée en présence de L-canavanine et la diminution très forte de PER observée avec la strychnine en mélange avec du sucre mais pas lorsque les stimuli sont dissociés, nous avons entrepris d'enregistrer l'activité de neurones gustatifs du proboscis sur des sensilles de différents types. Ce type d'observation est susceptible de nous donner des informations concernant le codage des informations gustatives à la périphérie.

Nous avons choisi les sensilles L5, S6 et I9 qui diffèrent par leurs profils de réponses. Les sensilles L5 comprennent 4 cellules, dont une répond aux sucres mais aucune autre n'a été répertoriée à ce jour comme sensible à des substances amères. Ces sensilles nous permettent d'observer des modifications des réponses aux sucres en l'absence d'activation concomitante d'une cellule sensible à l'amer. Les sensilles S6 comprennent 4 cellules également, dont l'une est sensible aux sucres et une autre répond aux substances amères. Ces sensilles nous permettent d'observer les réponses aux deux stimuli, présentés seuls ou en mélange. Enfin, les sensilles I9 ne comprennent que deux cellules, dont l'une est sensible à des sucres et l'autre à des substances amères (Hiroi et al., 2004). Ces sensilles sont intéressantes en particulier dans la perspective de l'ablation d'une cellule ou l'autre, car on peut ainsi observer l'action de mélanges sur une seule cellule gustative dépourvue de voisines.

III.- Mélanges binaires sucré-amer

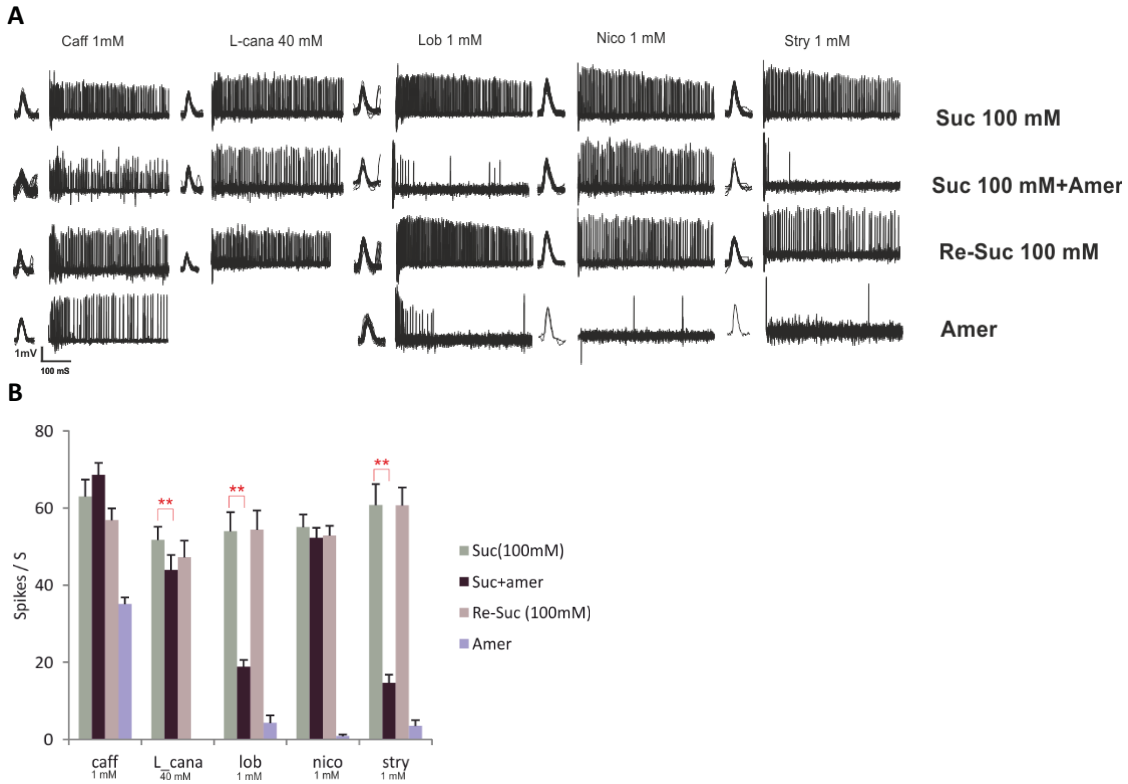


Figure 28 : Inhibition de la détection du sucre sous l'effet de molécules amères (sensille i9)

(A) Exemples de réponses aux enregistrements obtenus en stimulant des sensilles i9 sur des mouches témoin, w^{1118} . La séquence de stimulation était sucre, sucre+amer, sucre et amer. Le sucre testé était du saccharose 100 mM (suc). Les substances amère étaient la caféine 1 mM (caff), la L-canavanine 40 mM (L-cana), la lobeline 1 mM (Lob), la nicotine 1 mM (nico) et la strychnine 1 mM (stry). (B) Moyenne (+ SEM) du nombre total de potentiel d'action détectés pendant 1 s en réponse à ces stimuli ($n=7-10$). On observe une inhibition réversible de la réponse au sucre pour la lobeline et la strychnine, mais pas pour la nicotine ou la L-canavanine. La situation avec la caféine est moins claire car les enregistrements (A) suggèrent que deux neurones sont actifs.

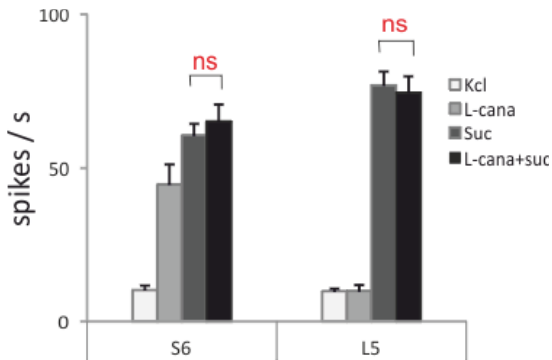


Figure 29 : Comparaison des réponses des sensilles S6 et L5 à la L-canavanine

Activité observée dans des sensilles gustatives L5 et S6 chez des mouches (w^{1118}) en réponse au saccharose (100 mM; suc) et à la L-canavanine (40 mM; L-cana) présentés isolément ou en mélange. Aucune inhibition de la réponse au sucre n'est observée (Mann-Whitney U Test $p = 0.721$ pour L5 et $P=0.153$ pour S6). $n=10$

Sur les sensilles I9 (Figure 28), nous avons obtenu une réduction statistiquement significative du nombre total de potentiels d'action émis par les cellules lorsque le saccharose 100 mM est mélangé à de la L-canavanine (40 mM), de la lobeline (1mM) ou de la strychnine (1 mM), mais pas pour la caféine (1 mM) ou la nicotine (1 mM). Cette inhibition de la L-canavanine n'est pas présente sur des sensilles S6 et L5 (Figure 29).

4. Discussion

Les observations que nous avons menées montrent que chez les drosophiles adultes, la réponse gustative au saccharose est inhibée de manière forte mais réversible par la strychnine mais pas par la L-canavanine. D'autres substances sont connues pour inhiber la réponse aux sucres comme la quinine (Meunier et al., 2003b), ou la lobeline (Figure 28), ou encore des alcaloïdes produits par les plantes (Chapman, 2003). Nous avons observé que cette inhibition de la réponse aux sucres avait une influence forte sur les réponses aux sucres dans des comportements alimentaires, que ce soit de la consommation sur 2 h ou l'extension du proboscis consécutif à une stimulation tarsale. Enfin, nos données suggèrent que cette inhibition de la sensibilité aux sucres est un mécanisme intrinsèque aux cellules sensibles aux sucres, puisqu'une inhibition comportementale est présente même lorsque une majeure partie des neurones sensibles à l'amer est éliminée par une ablation génétique.

Les observations que nous avons menées dans le cadre de ma thèse ont été intégrées dans un article rassemblant ces observations et celles d'autres membres du laboratoire (French et al., 2015b). Nos observations ont été confirmées en étendant le panel de substances testées et en utilisant d'autres constructions génétiques et gènes rapporteurs, notamment optogénétiques, qui ont permis de renforcer l'hypothèse de travail présentée ici, à savoir que les neurones sensibles aux sucres possèdent un mécanisme intrinsèque de sensibilité à des substances amères.

L'absence d'inhibition de la L-canavanine à l'égard de la détection du saccharose est intéressante et demanderait à être confirmée dans sa généralité. Cette propriété n'est pas intrinsèque à cette molécule puisque la L-canavanine a été notée comme inhibitrice de la détection des sucres chez *Schistocerca gregaria* (Chapman et al., 1991). On pourrait par exemple se demander quel est le statut de cette molécule chez les larves de drosophiles et si l'ablation génétique des neurones sensibles à l'amer aurait le même effet que chez les adultes.

C. Interactions sucres → amer [article 3]

Sugars suppress bitter taste in *Drosophila*

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1. Abstract

Bitter taste is not only useful to organisms to avoid ingesting noxious molecules, but it is also a limitation as it could prevent organisms to feed on nutritious food. While animals can overcome distaste by central mechanisms, bitter taste suppression can also occur directly at the periphery, within gustatory receptor neurons. In this work, we evaluated if 12 sugars could reduce the response of bitter-sensitive cells in flies. We first checked if flies could overcome the aversive taste of 4 bitter substances (denatonium, berberine, umbelliferone, caffeine) when mixed with a panel of 12 sugars, including nutritious and non-nutritious molecules. We observed that flies consume more of the bitter solutions with increasing concentrations of sugar. We then looked at the responses of taste sensilla on the proboscis of these flies, and observed that the responses to mixtures of bitter and sweet substances were lower than the responses to the isolated compounds. By separating the respective activities of the bitter- and of the sugar-sensitive gustatory neurons, we showed that sugars and bitter molecules have reciprocal inhibitory effects. While the inhibition of the bitter sensitive responses were about 25 % at the maximal concentration tested, the responses to sugars were diminished up to 60%. We confirmed these observations by recording from gustatory sensilla in which the sugar-sensitive neurons had been ablated, by means of a genetic construction. We show that these flies exhibit similar sweet inhibition than in the control flies. Lastly, we show here that a mixture of two sugars is more efficient than a sugar alone. These observations demonstrate that sweet inhibition also occur in flies and suggest that this inhibition properties are due to the gustatory neuron itself rather than due to interactions between gustatory neurons within the same sensilla.

2. Introduction

The sense of taste is crucial to quickly evaluate if food resources might be nutritious and if they contain noxious chemicals (Lindemann 2001; Yarmolinsky *et al.* 2009). This sensory system must deal with numerous molecules produced by plants, many of which are toxic (Ames *et al.* 1990). Most if not all plant secondary compounds such as alkaloids, plant-based phenols, flavonoids, isoflavones, terpenes, and glucosinolates taste bitter, acrid, or astringent and are aversive to the consumer (Drewnowski and Rock 1995; Bravo 1998). Although in few cases, bitter taste is tolerated or acceptable e.g. tea, coffee, wine, beer or bitter lemon (Ley *et al.* 2005), the bitterness of legumes and fruits is a problem in the food industry as it decreases acceptance of food. Bitterness deters consumers from ingesting beneficial phytonutrients which would lower their risks of suffering from cardiovascular diseases, obesity, diabetes or cancer (Peyrot des Gachons *et al.* 2009). As bitterness is a problem in the food industry, a number of debittering methods and natural or synthetic taste masking molecules are developed to diminish or eliminate the bitter taste (Drewnowski and Gomez-Carneros 2000; Ley 2008). Amongst these methods, the addition of sugar or sweeteners remains one of the simplest way to mask bitterness (Wilkie *et al.* 2013; Mennella *et al.* 2015) as sweetness at moderate to high intensities is generally suppressive of other basic tastes (Keast and Breslin 2002).

Most of these bitter compounds are aversive to insects, whether they are phytophagous (Schoonhoven *et al.* 1992; Schoonhoven and van Loon 2002; Chapman 2003), detritiphagous (Dethier 1976; Dethier and Bowdan 1992; French *et al.* 2015a and refs. therein), or blood feeders (Kessler *et al.* 2013; Kessler *et al.* 2014; Pontes *et al.* 2014). Numerous examples show that bitter substances inhibit sugar detection in insects. For example, in flies, alkaloids suppress sugar detection (Dethier and Bowdan 1989; 1992), and 1 mM quinine is enough to completely inhibit the peripheral detection of 35 mM fructose in *Drosophila* adults (Sellier *et al.* 2011). Such suppression is due to peripheral and central components. As in Vertebrates, the inhibitory effect of bitter chemicals depends upon satiety level, hungry insects accepting to feed on higher concentrations of bitter substances than satiated insects (Dethier 1976; Farhadian *et al.* 2012; Marella *et al.* 2012). This response is centrally regulated through conserved pathways between flies and mammals, e.g. neuropeptide and insulin-like signaling (Wu *et al.* 2005). In addition, local circuits fine tune the integration of taste information in the suboesophageal ganglion through GABA interneurons (Chu *et al.* 2014; Pool *et al.* 2014). At the periphery, the responses of sugar-sensitive neurons are inhibited by alkaloids (French *et al.* 2015b), an inhibition which might be mediated or facilitated by specific OBPs as shown by Jeong *et al.* (2013) with OBP49a.

Sweet suppression of bitter taste in insects is less documented. Simmonds *et al.* (1990) reported that the detection of azadirachtin is inhibited by sucrose in *Spodoptera littoralis* larvae, but that the detection of other alkaloids is not affected by increasing concentrations of sucrose. Inositol and

sucrose suppress the detection of sinigrin in *Mamestra configurata* and *Trichoplusia ni* larvae (Lepidoptera) (Shields and Mitchell 1995). Inositol (but not sucrose) masks the taste of caffeine in *Manduca sexta* larvae (Glendinning *et al.* 2000). More recently, Cocco and Glendinning (2012) showed that sweet suppression occurs in *M. sexta* larvae, when sucrose, inositol, or glucose is mixed with caffeine or aristolochic acid. Furthermore, they found that binary mixtures of sugars are more effective in suppressing bitter taste than single substances, and that sugars differ considerably in their capacity to mask bitter taste.

Here, we examine whether sugars could alter the detection of bitter substances in *Drosophila*. We selected four aversive compounds which differ by their deterency, *e.g.* denatonium > berberine > umbelliferone > caffeine according to Weiss *et al.* (2011). These aversive compounds were mixed with 12 sugar molecules, which differ in their sweetness and nutritive power according to Hassett (1948). We first recorded if short term consumption of flies was differently affected by these bitter substances according to the identity and the concentration of the sugar molecules. Interestingly, caffeine was the most inhibitory over the whole panel of sugars. We then asked if sugars could alter the response of bitter-sensitive neurons, by recording the electrophysiological responses from two i-type labellar sensilla which host only two cells of opposite modality (Hiroi *et al.* 2004). This makes easier to distinguish the activities from bitter- and sugar-sensitive cells within extracellular recordings. We observed that almost all sugars significantly inhibited the activity of bitter-sensitive cells at 100 mM. However, only four sugars (maltotriose, sucrose, glycerol and maltose) elicited strong responses in the sugar-sensitive cells in the presence of bitter substances. Lastly, we evaluated if bitter-sensitive cells could be inhibited by sugar-sensitive cells, by comparing the responses of flies with ablated sugar-sensitive cells to that of normal flies. We recorded the gustatory responses of i9 sensilla to mixtures of caffeine and theophylline with sucrose, glycerol and a mixture of the two. We found that both control and mutant flies showed a reduced response of their bitter-sensitive cells with 10 mM sucrose or 10% glycerol, and that a mixture of the two sugars was more effective than single sugars. Our observations demonstrate that most sugars exert a suppressive effect on the detection of bitter chemicals and that this inhibition is not dependent on the activity of sugar-sensitive cells.

3. Materials and Methods

Drosophila stock and transgenes. Flies were maintained on a standard axenic medium at 75% - 80% humidity, and 25°C± 1°C on a 16:8 light-dark cycle. Only females were used for electrophysiological tests. Both males and females were used for the behavioral tests. The flies tested were 2-6 d old. White type flies *w¹¹¹⁸*, *UAS DTI*, and *Gr5a-Gal4* flies were from Kristin Scott (UC Berkeley, USA).

Chemicals. All molecules were from Sigma Aldrich purchased at the highest grade. Bitter molecules were used at the following concentrations: 10 mM caffeine (CAF), 0.1 mM berberine chloride (BER), 0.1 mM denatonium benzoate (DEN), 10 mM theophylline anhydrous (TPH) and 10 mM umbelliferone (UMB). These concentrations were determined following pilot experiments which suggested that these molecules would elicit between 20 and 30 spike/s from i8 and i9 sensilla. The sugar molecules were sucrose (SUC), D- trehalose dehydrate (TRE), Inositol (INO), D-galacturonic acid (GALA), D-melezitose hydrate (MEL), D-maltose hydrate (MALT), maltotriose hydrate (3MALT), D-fructose (FRU), D-sorbitol (SORB), D-glucose anhydrous (GLU), glycerol (GLY), D-arabinose (ARA). The sugar molecules were used a 1, 10 or 100 mM (or 0.1, 1, 10 % for glycerol). All solutions were stored at -20°C aliquots and kept at 4°C for no more than one week. For electrophysiology, the solutions were completed with 1 mM KCl or 30 mM tricholine citrate (TCC), which served as an electrolyte and contributed to inhibit the activity from the water cell (Wieczorek and Wolff 1989). For behavioral observations, the solutions were completed with erioglauine 125 mg/100ml in order to improve the detection of the liquid levels within capillaries.

Behavioral assay. Assays were performed according to Sellier *et al.* (2011). Briefly, freshly emerged flies (1-2 d old) were transferred to fresh food for one day and starved for 20-22 h with access to water. Then, flies were divided into groups of 20 flies and transferred into a rectangular transparent plastic boxes (95x76 x15 mm, Caubère, France). Each box contained 5 capillaries (5 µl, Hirschmann Laborgeräte, VWR) which were disposed on a microscope slide with double sided sticky tape. Each capillary was filled with a test solution with a blue dye . Flies were kept in the dark for 2h at 25°C (Sellier *et al.* 2011). A box without flies was used as a control to monitor evaporation. A picture of each box was taken on a flatbed scanner at the beginning and at the end of an experiment, and the length of the liquid within each capillary was measured using ImageJ (Abramoff *et al.* 2004).

Electrophysiological recordings. Electrophysiological recordings were performed as in French *et al.* (2015b). Briefly, 2-6 d old flies were collected and briefly anesthetized on ice. They were immobilized on putty (yellow Patafix, UHU) placed on a cylindrical magnet, with fine stripes of tape, in order to expose sensilla of the labellum. A drop of electrocardiogram gel was disposed between the abdomen and a silver wire connected to the ground. Individual taste sensilla on the proboscis were stimulated by covering their tip during 2 s with a capillary electrode containing a stimulus dissolved in 1 mM KCl or 30 mM TCC. Stimuli were separated by a time interval of 2-5 min in order to avoid adaptation and contamination from the previous stimulus. The sensilla recorded were selected and named according to Hiroi *et al.* (2002). The electrical signals were amplified with a taste probe (Marion-Poll and van der Pers 1996), further amplified and filtered (x500 – 1000; 10-2800 Hz:

CyberAmp 320, Axon Instruments, USA), digitized at 16 bits, 10 kHz (DT9800, Data Translation, USA) and further stored and analyzed using a custom program, dbWave ([Marion-Poll 1996](#)).

Experiment 1. We asked if mixing these bitter molecules with sugars at different concentrations can reduce the consumption of flies and if specific mixture combinations were less deterrent than others. Groups of 20 flies were given a choice between 5 capillaries, 4 capillaries containing either of the sugar molecules (at the same concentration: 1, 10 or 100 mM; 0.1%, 1%, 10% for glycerol) mixed with a bitter substance (denatonium, berberine: 0.1 mM; umbelliferone, caffeine: 10 mM). Sugars were tested in three combinations: (a) fructose, melezitose, galacturonic acid and maltotriose, (b) sorbitol, inositol, glucose and trehalose, and (c) sucrose, glycerol, arabinose and maltose. A fifth capillary contained the same bitter substance that was mixed with the sugars, 1, 10 or 100 mM. As for the MultiCAFE test, each box contained 4 capillaries filled with either of these sugars mixed with one bitter substance and one capillary filled with the bitter substance. The sugars within each box were presented at the same dilution 1, 10 or 100 mM. Ten repetitions of each combination were tested (3 groups of 4 sugars (a,b,c) x 3 concentrations).

Experiment 2. In order to evaluate if mixtures of these molecules were detected independently by separate taste cells, we recorded the electrophysiological responses of i8 and i9 sensilla to the same molecules that were tested behaviorally. Only one series of concentrations was tested on a single sensillum in the following sequence: (a) bitter, (b) bitter + 1 mM sugar, (c) bitter + 10 mM sugar, (d) bitter + 100 mM sugar, (e) bitter, (f) 100 mM sugar. According to the results of experiment 1, i8 sensilla were stimulated with denatonium, berberine and caffeine, while i9 sensilla were stimulated with umbelliferone and caffeine. Each combination of sugar (n= 12) x bitter (n=4) was tested 10 times.

Experiment 3. In order to evaluate if sugar-sensitive cells participate to the suppression of the bitter taste, we performed a series of electrophysiological observations on i9 sensilla of flies in which the sugar-sensitive cells were genetically ablated. To do so, we crossed two parental lines respectively carrying Gr5a-Gal4 and UAS-DTI (Wang *et al.* 2004). These flies (hereafter denoted Gr5a > DTi or mutant flies) and their parents were tested with 10 mM caffeine or 10 mM theophylline mixed with increasing concentrations of glycerol (0.1, 1, 10%) or sucrose (1, 10, 100 mM). Such flies were also tested for a combination of sugars, by stimulating them with caffeine or theophylline mixed with sucrose and glycerol. Ten repetitions of each situation were recorded.

Data analysis. Data from the multiCAFE tests were measured and corrected for evaporation by measuring the volume of liquid lost in boxes without flies but prepared along with the other boxes in the same way. The corrected consumption was subjected to a two-ways ANOVA analysis.

Electrophysiology data were measured as number of action potentials emitted during the first second of each recording. Putative action potentials were detected by adjusting a threshold over a filtered version of the signal (a 30 points running median) and by extracting 60 points each time the signal crossed the threshold. The waveforms were subsequently sorted by interactive procedures under dbWave (Marion-Poll 1996; Meunier *et al.* 2003). As i-type sensilla are housing 2 gustatory cells and one mechanosensory cell, spikes were sorted in 3 classes on the basis of their amplitude and spiking frequency. As each stimulus series included the best stimulus for each cell, we could check the validity and consistency of our sorting procedures over all recordings obtained from the same sensillum. Data from experiment 3 were first tested with a Friedman's test of paired values and then with an ANOVA test. Data from experiment 4 were tested with Friedman and Dunn's multiple comparisons tests of paired values. The statistical values were computed using GraphPad Prism 6 (Friedman and Dunn's multiple comparison tests) or Statistica 10 (ANOVA analysis).

4. Results

Experiment 1

Flies given access to sugars mixed with a bitter substance consumed different amounts of the solutions depending on the combination of the 3 factors bitter molecule x sugar molecule x sugar concentration. Among the four bitter substances, 10 mM caffeine was the most inhibitory, while 0.1 mM denatonium, 0.1 mM berberine and 10 mM umbelliferone yielded similar levels of consumption (Figure 30 A). Across all experiments, flies fed significantly more of the solutions with increasing concentrations of sugars (Figure 30 B). Amongst sugars, maltotriose, glucose, sucrose were the most stimulatory, while sorbitol, galacturonic acid and arabinose were almost not consumed (Figure 30 C). Further comparisons are hampered by the fact that we used fixed combinations of sugars.

III.- Mélanges binaires sucré-amer

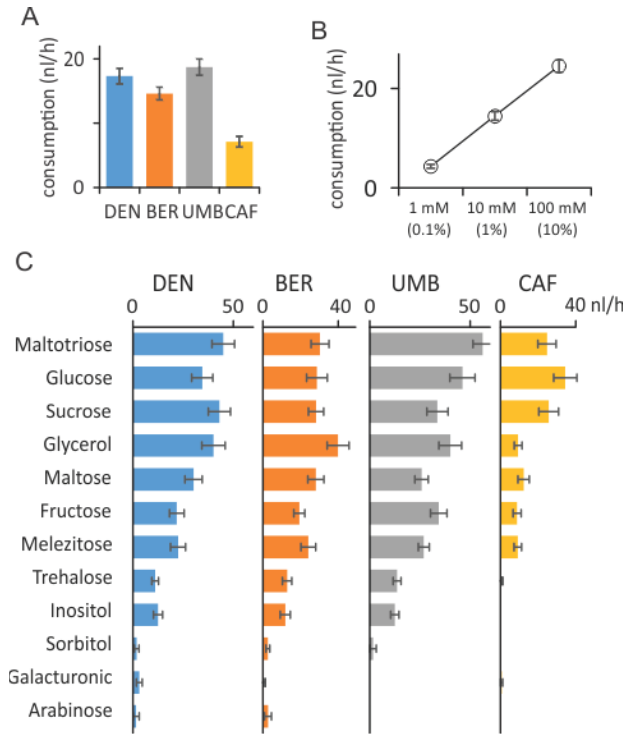


Figure 30: Sugar molecules reduce the effect of bitterness depending on their concentration and nature

Group of 20 flies were given access during 2 h to 6 capillary tubes containing either 0.1 mM denatonium (DEN), 0.1 mM berberine (BER), 10 mM umbelliferone (UMB) or 10 mM caffeine (CAF) mixed with 1 sugar (out of 12 tested) at 1 concentration (1, 10 or 100 mM). As each situation was reproduced 10 times, the entire set of data represents 10 x 12 sugars X 3 concentrations x 3 bitter substances. In this figure, the influence of the bitter substance, of the concentration of sugar and of the sugar are displayed separately by computing the respective averages over the entire set of data. **(A)** Influence of denatonium, berberine, umbelliferone and caffeine upon the consumption of sweet molecules (3 different concentrations 1, 10, 100mM for each of the 12 sugars tested or 1.0, 1, 10 % of glycerol). **(B)** Influence of the sugar concentration upon the consumption of bitter substances. **(C)** Influence of each sugar upon the consumption.

Experiment 2

We examined the responses from i8 and i9 sensilla to the same set of bitter and sugar molecules. These sensilla host only 2 neurons which allow us to separate the activities of the neurons within extracellular records obtained by capping the tip of one sensillum. The neural activities from i8 and i9 sensilla exhibited two classes of action potentials. One class was active in response to bitter substances and showed large spikes (1-5 mV). The other class was activated by sugars and showed spikes of smaller amplitude (0.3-1 mV) (Figure 31). These amplitudes could drift with the firing activity of the neuron and were smaller with mixtures (Figure 31). These two cells are called thereafter as L cell (large spike cell) and S cell (small spikes cell).

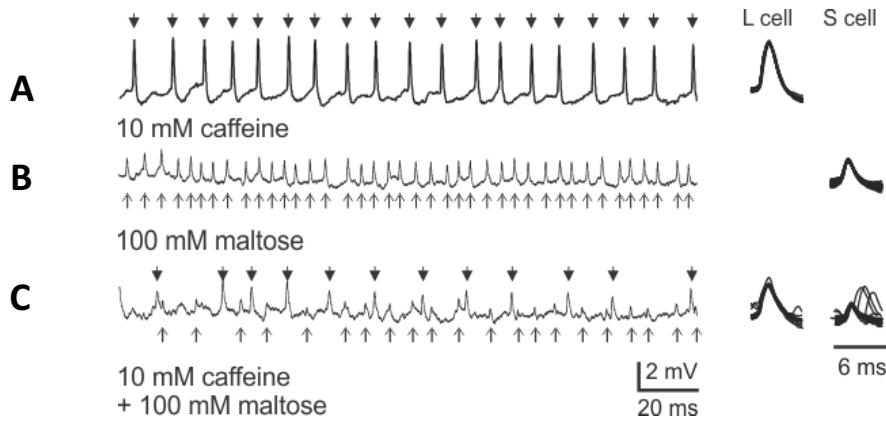


Figure 31: Maltose and caffeine activate two different classes of spikes in *i9* sensilla

Sample recordings obtained from *i9* sensilla using a tip-recording electrode. The central panel displays a selected portion of 200 ms of a 2 s recording. The spikes detected are pointed by an arrow placed over or below the trace. The right panel shows a superposition of the spikes detected in the central panel – the left column regroups spikes classified as “large” and the right column, the spikes classified as “small”. The vertical scale of these curves is indicated by a bar below the central panel. **(A)** 10 mM caffeine elicit spikes from one cell with large spikes (L cell). **(B)** 100 mM maltose elicit small amplitude spikes (s cell). **(C)** When 10 mM caffeine is mixed with 100 mM maltose, two classes of spikes are found which correspond respectively to the activities of the s and L cells. The spikes are notably smaller however than in the two previous situations. On the right panel, we see that some small spikes are followed by the firing of a larger spike, confirming that two cells are active at the same time.

In the absence of sugar, L cells responded with about 20 spikes/s to 0.1 mM denatonium and berberine, and with 25 spikes/s to 10 mM caffeine in *i8* sensilla; in *i9* sensilla, the cells respond to 10 mM umbelliferone with 28 spikes/s and to 10 mM caffeine with 35 spikes/s. The responses of S cells to 100 mM sugars were between 0 and 100 spike/s depending on the molecule.

We computed average responses over the whole set of recordings ($n = 3847$), in order to evaluate the effect of the bitter treatment on the responses (Figure 32 A). The influence of each bitter substance on the responses of L- and S-cells was quite similar. We also computed the responses of L- and S-cells to increasing concentrations of sugar. We observed a reduction of the firing activity of the L cells in relation to the sugar concentration of about 25% (Figure 32 B). Inversely, in the presence of bitter molecules, the responses of S cells to 100 mM sugars was reduced down to 55 % (*i8*) and 61% (*i9*) of the responses without sugars.

We analyzed the reduction of firing activity of L cells, induced by individual sugars by comparing the responses of the mixtures to the responses to the bitter substance alone. Almost all sugars exhibited an inhibitory activity at 100 mM (Figure 32 C). For denatonium, the largest reductions were recorded with maltotriose and melezitose. With berberine, maltotriose, glycerol, sucrose and maltose were the most inhibitor. Umbelliferone was mostly inhibited by maltose and galacturonic acid. With caffeine, the most efficient molecules were maltose, glycerol melezitose and galacturonic acid.

We also compared the responses of S cells toward 100 mM sugar and the responses to the mixture. First, the firing activities elicited by the sugars alone differ greatly with the molecules. Inositol and sorbitol elicit very few spikes, while maltose, maltotriose, sucrose, glycerol and arabinose are quite stimulatory.

In summary, stimulating i-type sensilla with a binary mixture of a bitter compound and a sugar induces most of the time a reciprocal inhibition of the respective responses of L-cell and S-cells.

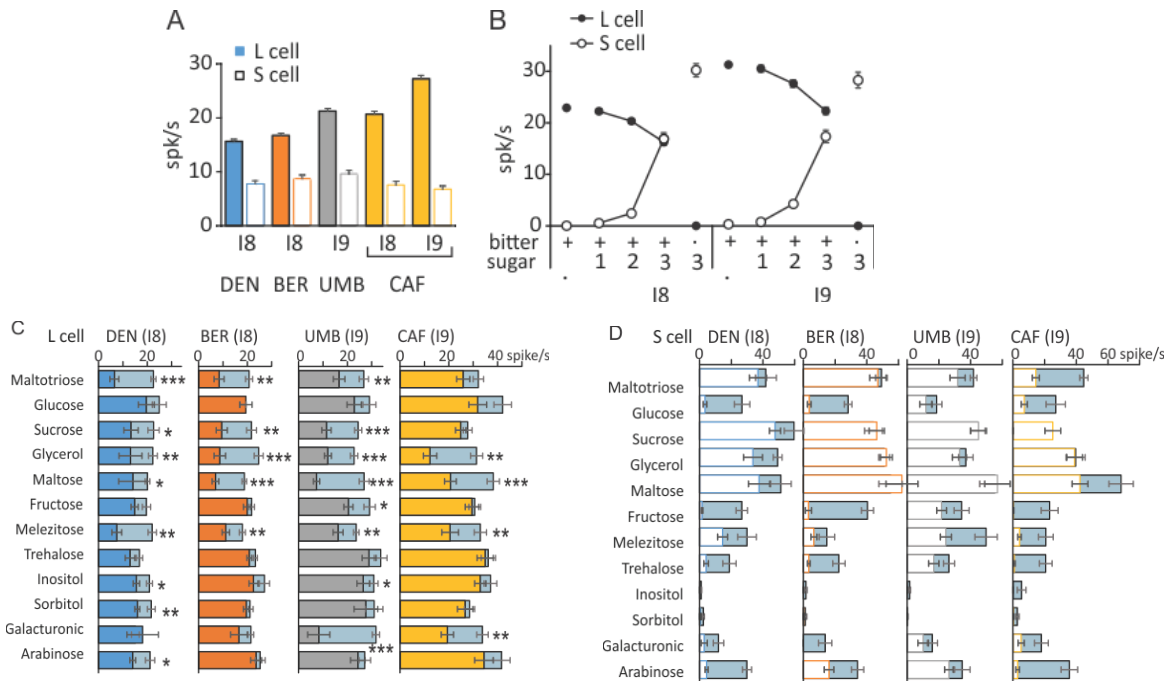


Figure 32: Electrophysiological responses of i8 and i9 sensilla to mixtures of bitter and sugar molecules at different concentrations

We used the same combination of mixtures of bitter and sugar stimuli to stimulate individual taste sensilla of the proboscis (i8 and i9) which house only 2 cells. In the same way as in figure 31, the average effects of bitter molecules, of the concentration of sugar and of each sugar is displayed in separate figures. We measured the activities of sugar-sensitive and bitter-sensitive cells and display here the number of spikes recorded during the first second of the recordings. The number of measures is n=10 for each combination of stimuli (4 bitter x 12 sugars x 4 sugar concentrations 0, 1, 10, 100 mM) (A) Effects of each bitter molecule upon the responses of cells within i8 and i9 sensilla. Filled bars represent the responses of bitter-sensitive cells (large spikes). The amplitude of the responses are slightly different. Caffeine was tested both in i8 and i9 sensilla. Empty bars show the average responses of sugar-sensitive cells (small spikes). (B) Effect of sugar concentration upon the responses of the bitter (black dots) and sugar (empty dots) cells. (C) Influence of the sugar molecules upon the responses of the bitter-sensitive cells to mixtures of denatonium, berberine, umbelliferone and caffeine. Colored bars (blue, orange, grey and yellow) represent the average responses in the presence of sugar. The blue-grey bars display the average response of the bitter-sensitive cells to the bitter substance alone. (D) Influence of the sugar molecules upon the responses of the sugar-sensitive cells in a mixture with denatonium, berberine, umbelliferone or caffeine (empty bars) or alone (blue-grey bars). Each bar or point represents the average of the number of spikes observed during the first second of the recordings. The brackets show the SEM. MANOVA tests were used to check the significant difference between the control condition (no mixture) and the response to the mixture.

Experiment 3

As the previous results rely upon an experienced observer to sort the spikes, we confirmed that sugars can inhibit bitter detection by using a genetic construction to eliminate the S cell. This was obtained by crossing flies carrying a promoter sequence of a sugar receptor, *Gr5a*, to drive the expression of GAL4, together with flies carrying as a reporter gene, the diphtheria toxin *DTI*. We used both parents (*Gr5a-Gal4* and *UAS-DTI*) as control lines and compared their response to the F1 progeny (*Gr5a > DTI*).

As found in the previous experiment, i9 sensilla responded to 10 mM caffeine with large amplitude action potentials. When caffeine or theophylline were mixed with increasing concentrations of sucrose or glycerol, the response of L cells decreased in the mutant flies as well as in the parental lines (Figure 33 A). The L cells responded with increasing spiking activity to higher sugar concentrations in the parental lines but not in the mutant flies (Figure 33 B).

We further tested a mixture of the two sugars, mixing glycerol with sucrose. We observed a significant reduction of the responses to caffeine and theophylline in the mutant flies as well as in the parents (Figure 33 C). The S cell showed a parallel increase of firing activity in the parental lines but not in the mutant.

III.- Mélanges binaires sucré-amer

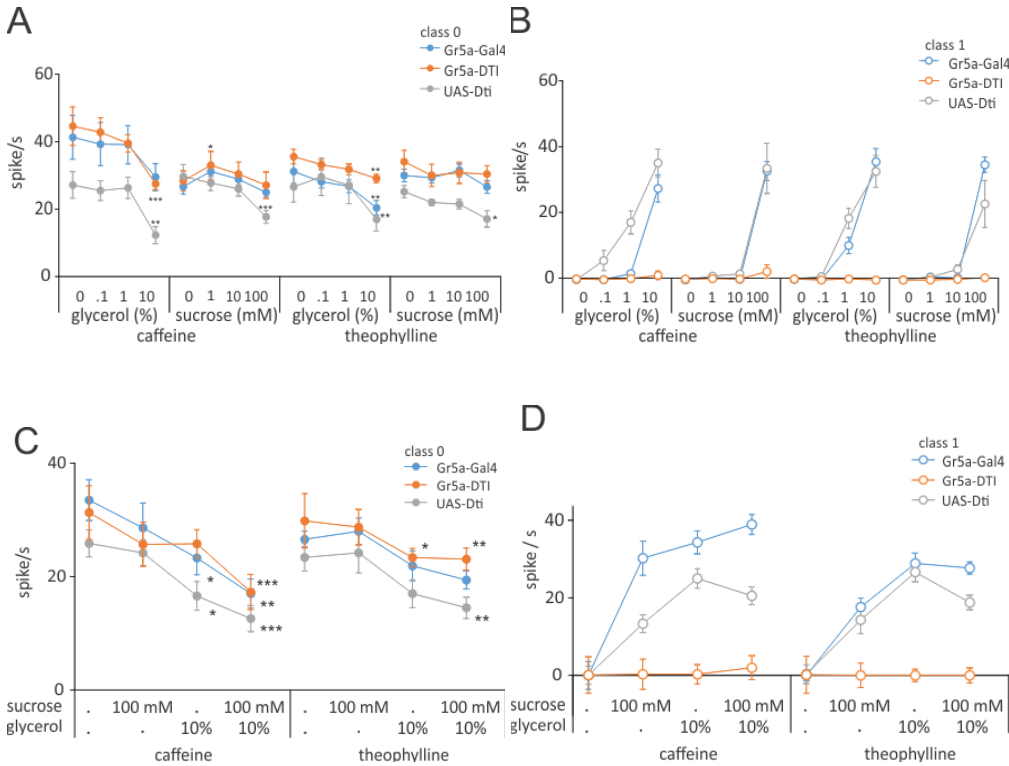


Figure 33: The responses of bitter-sensitive cells are inhibited by sugar in the absence of sugar-sensitive cells

In these two experiments, we compared the responses of bitter sensitive cells in the presence or absence of a sugar-sensitive cell towards (a) caffeine or theophylline mixed with different concentrations of sucrose and glycerol, or to a combination of these two sugars at a single concentration to seek for an additive effect. Sugar-sensitive cells were ablated using a genetic construction, by expressing the diphtheria toxin (DTi) into sugar sensitive cells expressing Gr5a. In the mutant flies (denoted Gr5a>DTi), the sugar sensitive cells were ablated while they were functional in the parental lines (denoted Gr5a-Gal4 and UAS-DTi). **(A)** Responses of bitter-sensitive cells (L cells) toward 10 mM caffeine or 10 mM theophylline mixed with glycerol (0, 0.1, 1, 10%) or sucrose (0, 1, 10, 100 mM). **(B)** Responses of sugar-sensitive cells to the same stimuli. **(C)** Responses of bitter-sensitive cells to caffeine or theophylline mixed with either 100 mM sucrose or 10% glycerol or to both sugars. **(D)** Responses of sugar-sensitive cells to the same stimuli.

5. Discussion

In this work, we evaluated if sugars could inhibit the response of bitter-sensitive cells in flies. We first checked if flies could overcome the aversive taste of 4 bitter substances when mixed with a panel of sugars. We observed that flies consume more of the bitter solutions with increasing concentrations of sugar. We then looked at the gustatory responses of taste sensilla on the proboscis, and observed that the responses to mixtures of bitter and sweet substances were in general lower than the responses to the isolated compounds. By separating the respective activities of the bitter and of the sugar sensitive cells, we showed that sugars and bitter molecules have reciprocal inhibitory effects. While the inhibition of the bitter sensitive responses were about 25 % at the maximal concentration tested, the responses to sugars were strongly diminished up to 60%. Furthermore, while it seems that

almost all sugar tested can exert an inhibition, the inhibition of the four bitter substances was very different according to the sugar molecules. Lastly, we show here that a mixture of two sugars is more efficient than a sugar alone.

This is not the first time that such observations are reported in insects but nevertheless sweet inhibition has not received much attention in insects. The most recent observations were made on the gustatory sensilla of larvae of a Lepidoptera, *Manduca sexta* (Cocco and Glendinning 2012). In this work, the authors used a short term biting assay to monitor the behavioral responses towards tastants. The behavioral assay used here is running over 2 h, which opens the possibility that the results observed are due not only to inhibition occurring at the periphery, but also by post-ingestive evaluation. A useful complement to this study would be to test proboscis extension responses or to limit the observation period to a shorter time. This might be one reason why the behavioral responses to the range of sugars tested differs from the electrophysiological observations, as the two set of data concern very different time scales, 2 h for the behavior and 2 s for electrophysiology.

Within the sweet/bitter mixtures, each neuron showed a reduced activity. While bitter inhibition has been reported many times in insects and in *Drosophila* ((French *et al.* 2015a), sweet inhibition has never been observed in the flies. We took advantage of a specific feature of some taste sensilla of the proboscis that host only 2 gustatory neurons to analyze in more detail how sweet inhibition happens. Although this preparation is more favorable as regards spike separation, spike detection and sorting remains difficult in *Drosophila*. One of the reasons is that the amplitudes of the spikes fired from a given neuron vary with time and with the spiking frequency of the neuron (Fujishiro *et al.* 1984). Another reason is that the background noise of the recordings vary from fly to fly. Lastly, although we assumed that 2 neurons only are present in i8 and i9 sensilla, it is still possible that some variability occurs. In this experiment, we did not find much variability in the number of cells active. However, this may be specific to our laboratory strain since Weiss *et al.* (2011) reported that one of the sensilla tested here was quite variable in their hands, and that additional sensilla were present on the proboscis of their strain.

We confirmed that reciprocal inhibition occurred by ablating one of the taste cells within i9 sensilla and by showing that sugars do inhibit bitter detection in the absence of S cells. This result is important for two reasons. First, it confirms the observations we made, which were based on manual sorting of spikes from control insects. Second, it also means that this inhibition is not due to an interaction between gustatory cells of the same sensillum as suggested by Cocco and Glendinning (2012). While the demonstration made here is less comprehensive than previously done for bitter inhibition (French *et al.* 2015b), this suggests that lateral interactions within neurons of the same sensilla do not represent the main mechanism by which sweet or bitter inhibition occur. While bitter

inhibition can be partly explained or facilitated by OBPs ([Jeong et al. 2013](#)), especially for bitter substances which are amphiphilic and poorly water soluble, such a mechanism does not seem likely for sugars which dissolve really well in water. One possibility is that bitter and sweet molecules could associate to the gustatory receptors and thus diminish their responsiveness.

Our observations demonstrate that peripheral interactions occur in the gustatory system of flies and that these inhibitory interactions are quite significant. Sweet inhibition of bitter taste makes sense for nutritious sugars as it could allow insects to balance the benefit from ingesting nutritive food with the risk of ingesting noxious chemicals. However, similar inhibitions are observed with non-nutritious sugars such as arabinose. Therefore, the adaptive value of such interactions is probably limited. Further observations with a larger panel of sugars and molecules would be required to find more efficient bitter-masking molecules. Such molecules would be quite useful in crop protection, especially in “attract and kill” strategies strategy ([Mangan and Moreno 2007](#); [Witzgall et al. 2010](#); [Reisenman et al. 2016](#)), where they could contribute to mask noxious molecules.

6. Acknowledgments

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D. Discussion

Dans ce chapitre, nous avons rassemblé des expériences qui montrent que les substances amères et sucrées exercent des influences réciproques dans des mélanges binaires. Dans une première partie, nous avons étudié l'influence de substances amères comme des alcaloïdes et la L-canavanine sur les réponses au saccharose. Nous avons contribué à mettre en évidence que certaines de ces substances ont un effet inhibiteur très marqué sur les réponses au saccharose, alors que d'autres, comme la L-canavanine, n'ont quasiment aucun effet. En utilisant des constructions génétiques permettant de pratiquer une ablation des cellules sensibles à l'amer, nous avons pu montrer que cette inhibition de la détection des sucres ne requerrait pas la présence de cellules sensibles à l'amer et que très probablement, cette inhibition était une propriété des cellules sensibles au sucre.

Dans une deuxième partie, nous avons réalisé l'expérience inverse, afin de savoir si des sucres sont capables d'inhiber la réponse à 4 substances amères (berbérine, ombelliférone, caféine, dénatonium). Nous avons en effet observé que certains sucres avaient un effet plus prononcé sur la réponse aux substances amères et surtout qu'un mélange de deux sucres était plus efficace que des sucres isolés. Nous avons également pratiqué des ablations génétiques des cellules sensibles aux sucres afin de voir si cette inhibition était liée à une interaction entre les cellules activées par les sucres et les cellules répondant à l'amer. Nos résultats montrent que ce n'est pas le cas, en tout cas pas dans les conditions testées.

Bien que nous ayons pu démontrer des interactions négatives dans les deux situations, chez la drosophile, l'effet inhibiteur des substances amères sur la détection des sucres est beaucoup plus prononcé que l'inverse. Cette situation contraste avec l'expérience commune chez l'homme où les sucres sont assez efficaces pour supprimer le goût amer. Cette différence est sans doute liée à des différences dans les récepteurs protéiques impliqués dans ces perceptions. De manière plus générale, il est possible que les insectes ne puissent pas se permettre d'ingérer des substances toxiques même si les aliments sont très nutritifs, alors que chez l'homme dont le régime alimentaire est omnivore, l'incidence de la consommation des substances amères testées pourrait être moins importante.

IV. Modulation de la physiologie des récepteurs et détection des sucres

A. Un neuropeptide qui influence la détection du sucre

Beaucoup de neuropeptides qui ont été identifiés chez les insectes ont été caractérisés chez la drosophile (Nüssel, 2002). La drosophile produit plus de 60 peptides (de Haro et al., 2010). La plupart des neuropeptides sont produits par des interneurons et des cellules neurosécrétrices ou endocrines du système nerveux central ou de la chaîne ventrale. Ils peuvent agir soit comme des hormones, des neurotransmetteurs ou des neuromodulateurs (Nüssel, 2002). Ces peptides sont impliqués dans de nombreuses fonctions comme l'horloge circadienne, le traitement olfactif, l'apprentissage, la cour, la mue, etc. (Taghert and Veenstra, 2003; Kim et al., 2006; Nüssel and Winther, 2010).

La leucokinine (LK) est une neurohormone qui contribue à la régulation de l'eau et l'homéostasie des ions chez les insectes (Taghert and Veenstra, 2003). Elle induit la sécrétion de fluide dans les tubes de Malpighi et a des effets myotropes sur le tube digestif postérieur (Gäde, 2004). Chez la drosophile, cette neurohormone est connue sous diverses dénominations (Tableau 4).

Tableau 4 : Synonymes de la leucokinine (<http://flybase.org/reports/FBgn0028418.html>)

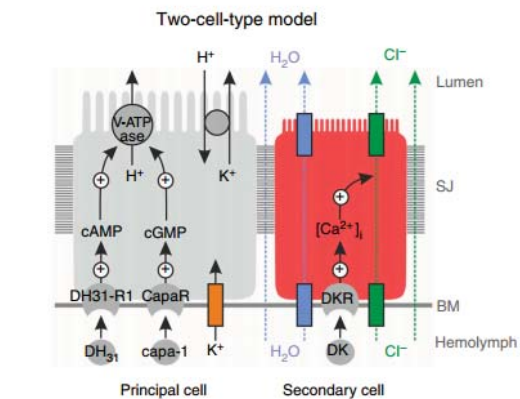
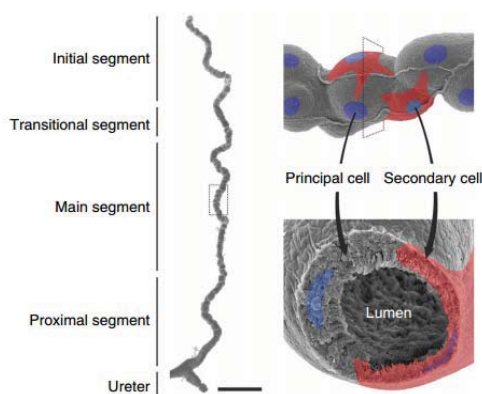
Symbole	Publication
CG13480	Hewes and Taghert, 2001, Yew et al., 2009, Perkins et al., 2009, Wegener and Gorbashov, 2008, Cammarato et al., 2011, Asahina and Anderson, 2013
DLK	O'Donnell and Spring, 2000, Baggerman et al., 2002, Pollock et al., 2000, Terhzaz et al., 1999, Nüssel, 2002
Drm-KIN	Van den Broeck, 2001
LCK	Baggerman et al., 2005
leucokinin	Santos et al., 2007, Itskov and Ribeiro, 2013, Liu et al., 2015
Leucokinin	Herrero et al., 2003, Wegener and Gorbashov, 2008, Veenstra et al., 2008, Japanese National Institute of Genetics, 2012, Itskov and Ribeiro, 2013
Leuk	Al-Anzi et al., 2010
leukokinin	Melcher and Pankratz, 2005
LK	Hewes et al., 2003, Herrero et al., 2007, Hewes et al., 2006, Park et al., 2008, Johnson et al., 2005, Johnson et al., 2008, Hector et al., 2009, Cognigni et al., 2011, Nüssel and Winther, 2010
Lk	Gauthier and Hewes, 2006, Terhzaz et al., 2010, Asahina and Anderson, 2013, Luo et al., 2013, Liu et al., 2015
lk	de Haro et al., 2010, López-Arias et al., 2011
pp	Radford et al., 2002a, Radford et al., 2002b, Terhzaz et al., 1999

Comme les autres neurohormones, la leucokinine agit sur un récepteur de type GPCR. Chez la drosophile, environ 200 GPCRs ont été annotés et 50 d'entre eux pourraient avoir comme ligand une protéine ou une neurohormone (Hauser et al., 2006). Le récepteur à LK est identifié chez la drosophile (Al-Anzi et al., 2010) (Tableau 5).

Tableau 5 : Synonymes pour le récepteur à la leucokinine (<http://flybase.org/reports/FBgn0035610.html>)

Symbole / nom	Publication
DLKR	Radford et al., 2002
dLKR	Southall et al., 2006
Lkr	Quinones-Coello, 2007, Bowser and Tobe, 2006, Caers et al., 2012, Denholm et al., 2013
LK-R	Johnson et al., 2008, Hector et al., 2009
LkR	Wang et al., 2004
lkr	Al-Anzi et al., 2010
LKR	Allan et al., 2005, Cardoso et al., 2012, Van Bortle et al., 2015
Drosokinin receptor	Radford et al., 2002
Kinin receptor	Caers et al., 2012
Leucokinin receptor	Radford et al., 2002, Veenstra et al., 2008, Allan et al., 2005
leucokinin receptor	Veenstra, 2009, Johnson et al., 2008, Al-Anzi et al., 2010, Cardoso et al., 2012, Itskov and Ribeiro, 2013

A



B

Figure 34 : Mécanismes du transport d'ions et d'eau dans le tube de Malpighi (Halberg et al., 2015)

(A) Organisation du tube de Malpighi et disposition des cellules principales et secondaires. Barre d'échelle : 100 mm (B) Modèle classique de fonctionnement des deux types de cellules. Le transport de fluides est alimenté par une ATPase de type V (V-ATPase) localisée dans la membrane apicale des cellules principales qui excrète des ions K⁺ dans la lumière du tube. Alors que transport de l'eau et des ions chlorure implique des voies intercellulaires ou intracellulaire (flèches vertes), la kinine (DK) active le transport d'ions chlorure par une voie intracellulaire dans les cellules accessoires. Ce mouvement d'ions chlorure active un transfert d'eau qui passe au travers des cellules ou entre les cellules. Le récepteur à la kinine (DKR) est localisé uniquement dans les cellules accessoires. Cette illustration est une reproduction de la figure 1 de Halberg et al. (2015).

Chez toutes les espèces d'insectes étudiées, des kinines activent un transport d'ions chlorure et d'eau au niveau du tube de Malpighi (Pannabecker et al., 1993; Cabrero et al., 2014). Les cellules responsables de ce transport sont les cellules secondaires (ou étoilées) (Dow, 2012), qui jouxtent les cellules principales qui sont elles-mêmes responsables de l'excrétion d'ions K⁺. Ces cellules sont la cible de plusieurs neuropeptides, mais les récepteurs à la kinine sont généralement limités aux cellules accessoires (Radford et al., 2002; Lu et al., 2011) (Figure 34). Ces cellules sont la cible de différents

neuropeptides chez la plupart des insectes, à l'exception des Coléoptères chez lesquels seule une petite fraction de ces cellules sont sensibles à ces neuropeptides (Halberg et al., 2015).

D'autres cellules épithéliales sont impliquées dans des phénomènes de transport comme les cellules du tube digestif moyen et les cellules accessoires des sensilles externes. En effet, les cellules épithéliales accessoires des sensilles des insectes se transforment en cellules excrétrices après la mise en place des structures cuticulaires, et sécrètent le liquide intrasensillaire dans lequel baignent les dendrites des neurones chimiosensoriels. Ce liquide est riche en ions K⁺ et la membrane apicale de ces cellules comporte des pompes V-ATPases (Sollai et al., 2008; Wieczorek et al., 2009). Un des rôles de cette excrétion serait de créer un potentiel transépithélial qui facilite la transduction dans les neurones sensoriels (Küppers and Thurm, 1979; Sollai et al., 2008). Compte tenu de cette analogie de fonctionnement, il est possible que les neuropeptides qui agissent sur le transport épithélial dans le tube de Malpighi aient un effet également sur ces cellules accessoires et influencent ainsi la sensibilité des neurones chimiorécepteurs.

Quelques observations suggèrent que les sensilles chimiosensorielles sont sensibles à la kinine. Par exemple, López-Arias et al. (2011) ont montré qu'en bloquant la production de potentiels d'action dans des neurones centraux produisant de la kinine, le comportement alimentaire de drosophiles adultes est modifié, en particulier à l'égard du tréhalose et de quelques substances amères (Figure 35).

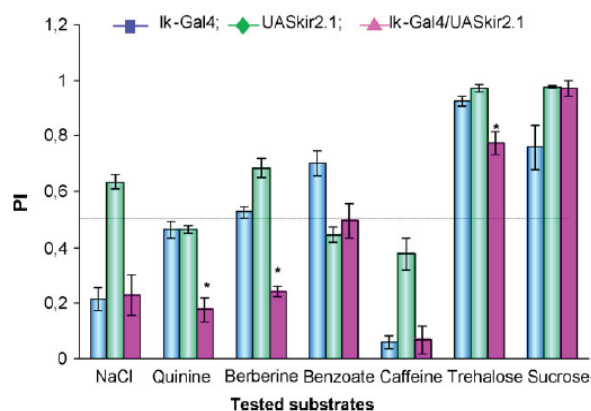


Figure 35 : Influence de l'inactivation de neurones LK sur comportement de choix à des sucres et substances amères (López-Arias et al., 2011)

La préférence alimentaire (test binaire avec des colorants bleu et rouge) a été mesurée en présence de saccharose 2 mM additionné de NaCl 100 mM, berbérine 1 mM, denatonium 0.25 mM, caféine 6 mM versus du saccharose 2 mM, ou du tréhalose 25 mM ou du saccharose 2 mM versus de l'eau. La préférence est quantifiée par un index de préférence (1 = appétitif, 0 = répulsif). Ces données suggèrent que les neurones LK sont impliqués dans la perception du tréhalose, de la quinine et de la berbérine (López-Arias et al., 2011).

Par ailleurs, il a été montré également que l'inactivation du récepteur à la kinine (LKR) dans des neurones centraux et des ganglions ventraux perturbe la régulation de la prise de nourriture chez des mouches adultes (Al-Anzi et al., 2010). Il y a donc dans la littérature, des données expérimentales qui suggèrent que les kinines sont impliquées dans la régulation des voies liées à la perception chimiosensorielles, au niveau central et/ou périphérique.

Ces éléments nous ont incités à répondre favorablement à une demande de collaboration de la part du professeur V. Pietrantonio, Professor l'université Texas A&M (USA), qui souhaitait évaluer l'effet d'analogues de kinines chez le moustique. Ces analogues de kinines avaient été synthétisés par le Prof. Nachman. Ces analogues sont à la fois compatibles avec le site accepteur du récepteur LKR (Taneja-Bageshwar et al., 2008) et plus stables que le ligand naturel (Taneja-Bageshwar et al., 2009). La durée de vie des neuropeptides naturels est très courte (Taghert and Veenstra, 2003), en particulier à cause de peptidases qui les dégradent rapidement, dans la membrane à proximité des récepteurs ou dans l'hémolymphe (Isaac et al., 2009). Les molécules analogues peuvent être soit des peptides synthétiques, soit des molécules non peptidiques (Kaczmarek et al., 2010; Nachman and Pietrantonio, 2010). Les molécules que nous avons testées (Figure 36) comportent des analogues d'acides aminés β à des sites attaqués par les peptidases (Zubrzak et al., 2007), ce qui leur confère une durée de vie plus longue *in vivo*. De manière générale, ces neuropeptides représentent une piste intéressante pour mettre au point des insecticides avec de nouveaux modes d'action (Nachman et al., 2011).

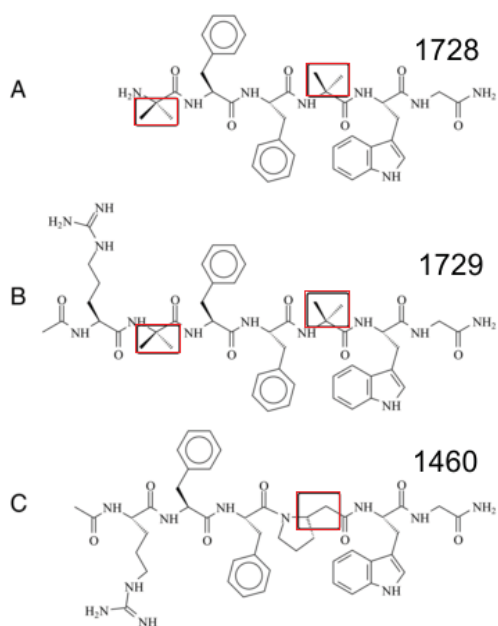


Figure 36 : Structure des analogues de kinine testés (Kwon et al., 2016)

Les trois analogues sont (A) K-Aib-1 ou 1728, (B) K-Aib-3 ou 1729 et (C) K- β A-1 ou 1460 (Taneja-Bageshwar et al., 2008). Les parties non-naturelles de ces molécules sont entourées d'un rectangle rouge.

Dans ce qui suit, je présenterai les expériences que j'ai réalisées sur *Aedes* pour valider l'action de ces analogues sur les sensilles gustatives de cet insecte, puis les observations entreprises sur *Drosophila* avec ces mêmes analogues.

B. Effets d'analogues de kinine sur la détection de saccharose chez *Aedes aegypti* (L.) [article 4]

1. Introduction

Aedes aegypti (L) (Culicidae- Diptera) est considéré comme le vecteur principal de la dengue. Il peut transmettre le virus zika comme d'autres moustiques du genre *Aedes* (Mousson et al., 2005; Morrison et al., 2008). Cette transmission peut être effectuée par la femelle au moment qu'elle se nourrit sur le sang dont elle a besoin pour la production d'œufs (Morrison et al., 2008). Chez cet insecte, les neuropeptides diurétiques augmentent la sécrétion de l'urine primaire par le tube de Malpighi et augmentent les contractions du tube digestif postérieur, qui aident à l'excrétion des fluides (Coast, 1996). Chez *A. aegypti*, trois kinines endogènes (aedeskinin I-III) agissent comme hormones diurétiques sur les cellules étoilées du tubule de Malpighi (Veenstra et al., 1997; Lu et al., 2011). Lorsque nous avons commencé ce travail, nos collègues avaient entamé des observations suggérant que la consommation de liquide sucré était diminuée après ingestion de solution contenant un analogue de kinine (Figure 36). Nous avons donc entrepris d'identifier les soies gustatives accessibles à l'enregistrement sur les tarsi et sur le proboscis (Figure 37).

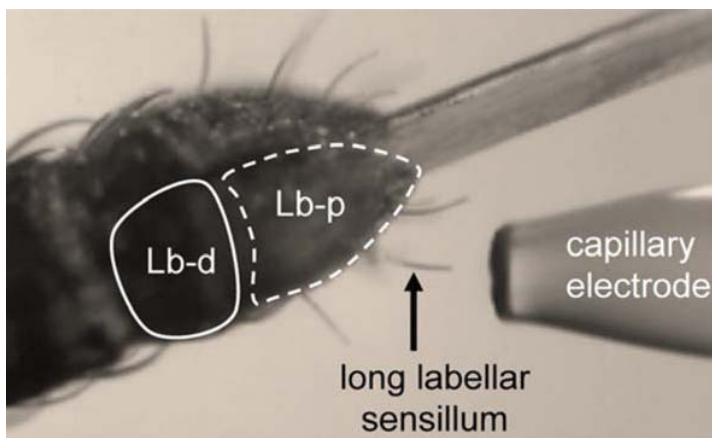


Figure 37 : Sensilles de l'extrémité distale du proboscis testées et électrode de stimulation

Nous avons testé les réponses des sensilles situées sur la partie distale du labium (Lb-p). Sur la photo, on voit le labium d'où émergent les stylets. Les soies courbes sont les sensilles testées dans ce travail. A droite, en bas, apparaît l'extrémité d'un capillaire de stimulation dont l'ouverture fait environ 10 μm . (Kwon et al., 2016: figure S5A)

Chez *A. aegypti*, comme chez d'autres insectes hématophages, les sensilles gustatives répondent à la fois à des stimuli généraux comme les sucres et les sels et à des stimuli caractéristiques de leur hôte vertébré, comme l'ATP (Galun et al., 1963). Les sensilles gustatives ont une structure externe similaire à celle des autres insectes, avec un pore terminal, et les neurones gustatifs du labellum se projettent dans le ganglion sous-oesophagien (Ignell and Hansson, 2005). Les adultes se nourrissent en partie sur le nectar des fleurs et sont capables d'exprimer des choix comportementaux en fonction des

sucres et de composés secondaires des plantes comme la quinine (Ignell et al., 2010). Ces neurones gustatifs présents sur le labellum et les tarsi expriment des récepteurs membranaires appartenant à la famille des Grs (Sparks et al., 2013) ainsi que des récepteurs Irs, des TRP et des OBPs (Sparks et al., 2014).

Les expériences que nous avons réalisées ont consisté à mélanger un des analogues de kinine à la solution test. Nos collègues ayant noté que les moustiques pouvaient exprimer une réaction de rejet actif en présence d'une goutte sucrée traitée, nous avons cherché à évaluer si la présence de kinine activait une cellule différente que celle qui répondait au sucre.

2. Résultats

Afin de comparer les réponses électrophysiologiques aux observations comportementales, nous avons stimulé les sensilles avec des solutions contenant du saccharose 300 mM et un de des analogues de kinines (1728, 1729 ou 1460) aux concentrations de 0, 0.01, 0.1 et 1 mM. Nous avons obtenu les insectes d'un élevage d'*A. aegypti* maintenu à l'Institut Pasteur par le Dr Lambrechts.

Nos enregistrements montrent une réduction significative du nombre de potentiels d'action en présence de 1 mM d'analogue, mais pas aux doses moins élevées (Figure 38). L'analogue 1728 semble plus efficace que les deux autres analogues. Cette inhibition est immédiate et concerne aussi bien la partie phasique que la partie tonique des réponses (Figure 38 C). Dans tous ces enregistrements, une seule classe de potentiels d'action était visible, ce qui suggère que seules les cellules sensibles au sucre répondent, même en présence d'analogue.

Nous avons également réalisé des enregistrements pour évaluer si la réponse au saccharose était affectée par le colorant bleu d'Evans (utilisé en comportement) ou par le tricholine citrate (TCC 30 mM). Aucun de ces composés ne semble altérer la détection du sucre dans ces sensilles longues du proboscis (Figure 39).

IV.- Modulation de la physiologie des récepteurs et détection des sucres

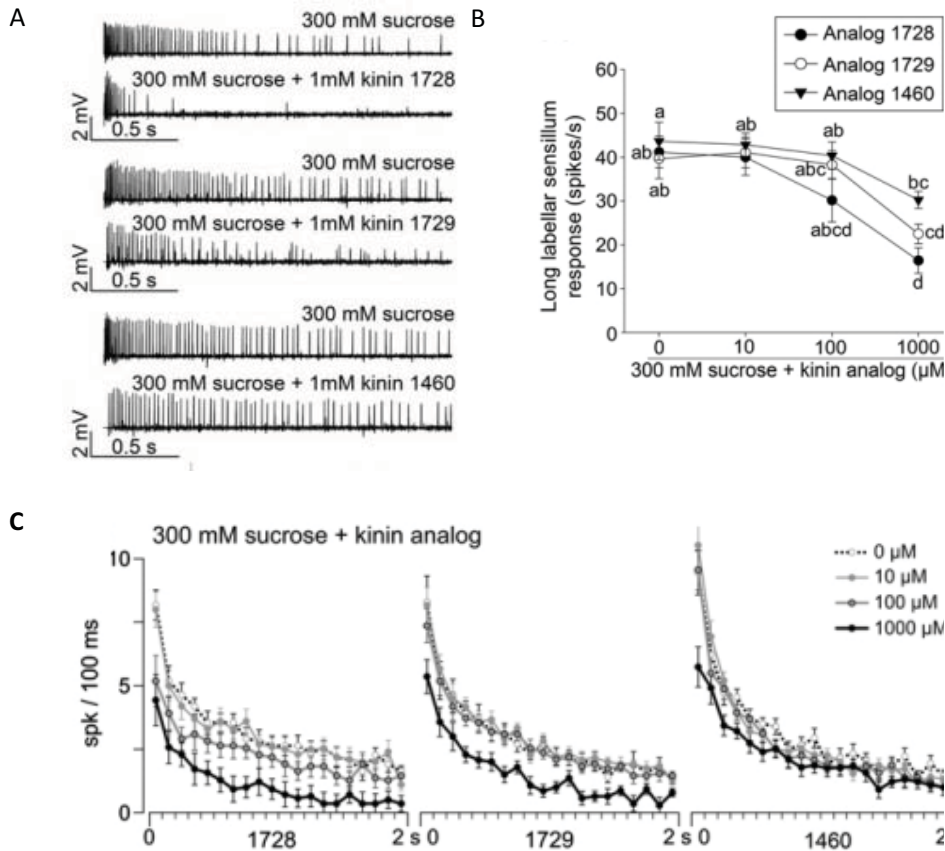


Figure 38 : Réponses de sensilles gustatives du proboscis à un mélange de saccharose et d'analogues de kinine chez des femelles d'*Aedes aegypti*

(A) Exemples d'enregistrements obtenus sur les sensilles longues du proboscis de femelles d'*A.aegypti* en réponse à du saccharose 300 mM en mélange avec un analogue de kinine (1728, 1729 et;1460). (B) Moyennes des réponses observées en fonction de la dose d'analogue (0-10-100-1000 μM). Une inhibition significative est observée à partir 10 μM pour les deux analogues 1728 et 1729 ; cette inhibition apparaît à 1 mM pour 1460 ($n=10-22$). (C) Dynamique temporelle des réponses obtenue en mesurant le nombre de potentiels d'action sur des intervalles de temps de 100 ms (moyenne \pm SEM). (Kwon et al., 2016: figure 3)

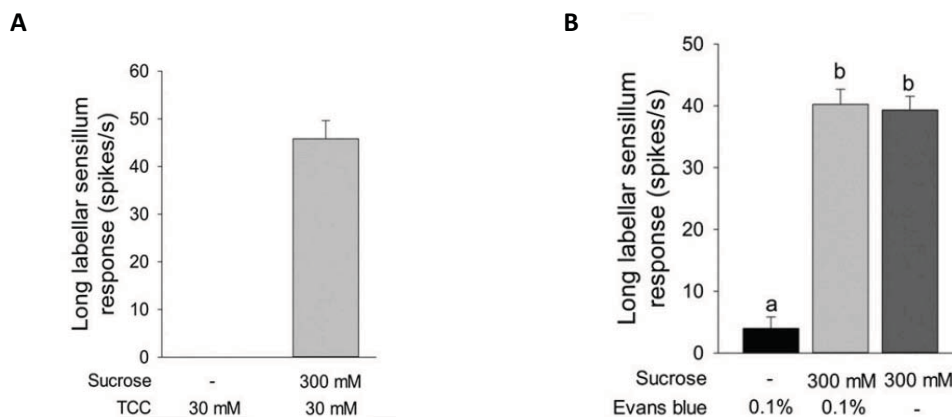


Figure 39 : Réponses électrophysiologiques au tricholine citrate et au bleu d'Evans

(A) Réponses au tricholine citrate (TCC 30mM) et saccharose 300 mM ($n=6-8$). (B) Réponses au bleu d'Evans (0.1 %) avec ou sans saccharose (300 mM) et saccharose seul ($n=12$). (Kwon et al., 2016: figure S5 B, C)

3. Discussion

Nos observations démontrent que l'effet inhibiteur des analogues de kinine sur les réponses comportementales au saccharose chez *A. aegypti* est lié à une réduction de la sensibilité périphérique. Cet effet est dose-dépendant et immédiat. Il conduit, au moins pour l'analogue 1728, à une réduction de plus de 50% de la réponse au sucre à la dose de 1 mM. Dans les enregistrements réalisés, nous avons détecté l'activité d'une seule cellule, à la fois en tenant compte de la forme des potentiels d'action et de leur occurrence temporelle (Meunier et al., 2003a). Ceci suggère que dans les sensilles étudiées, les analogues de kinine diminuent la sensibilité aux sucres mais n'activent pas de cellules sensibles aux produits amers.

Ces résultats n'expliquent pas le comportement aversif actif décrit par nos collègues lorsque les femelles sont en présence d'une solution mélangée à un analogue de kinine. Il serait utile de compléter nos observations sur d'autres sensilles gustatives, notamment des tarsi, soit en électrophysiologie, soit par l'observation d'extension du proboscis si ce comportement pouvait être étudié chez le moustique. Il semble établi cependant que les neurones gustatifs de ce moustique ainsi que les cellules accessoires expriment un récepteur aux kinines, récepteur probablement de type GPCR. Il aurait été intéressant de pouvoir confirmer cette hypothèse en effectuant des enregistrements sur les insectes traités au RNAi dirigé contre le récepteur LKR afin de confirmer son rôle au niveau des sensilles gustatives.

C. Effets d'analogues de kinine sur les réponses gustatives chez la drosophile [article 5]

1. Introduction

Chez les insectes, le métabolisme de l'eau et des ions est régulé en grande partie par les tubes de Malpighi et par le tube digestif postérieur. Un grand nombre de neuropeptides, appelés hormones diurétiques ou antidiurétiques, agit sur l'excrétion des tubes de Malpighi et la réabsorption d'eau et d'ions par le tube digestif. Chez la drosophile, 4 gènes codant pour des hormones diurétiques ont été identifiés : *Dh*, *Dh31*, *lkr* et *capa* (Taghert and Veenstra, 2003). La portion principale des tubes de Malpighi comprend au moins deux types de cellules, les cellules principales et les cellules étoilées. Alors que DH I, DH II, CAPA-1 et CAPA-2 agissent sur les cellules principales, notamment en stimulant la production de NO, les leucokinines agissent sur les cellules étoilées en augmentant la concentration intracellulaire de Ca⁺⁺, ce qui stimule la conductance des ions Cl⁻. Chez la drosophile, *lkr* est traduit en une seule protéine alors que chez *Aedes aegypti* ce gène est traduit en 3 peptides différents et que chez d'autres insectes, jusqu'à 8 peptides ont été identifiés (Taghert and Veenstra, 2003). La leucokinine de Drosophile a été isolée et représente la plus longue leucokinine identifiée (Terhzaz et al., 1999). De toutes les hormones diurétiques, la leucokinine semble être la plus efficace, agissant à des doses de 0.1 nM alors que CAPA-1, CAPA-2 et Dh31 agissent à des doses de 1 nM ou plus (Taghert and Veenstra, 2003).

Outre le rôle important des leucokinines dans la diurèse, ces hormones pourraient jouer un rôle dans la perception olfactive et gustative (López-Arias et al., 2011), ainsi que dans le contrôle de l'alimentation (Al-Anzi et al., 2010). Des kinines sont aussi impliquées dans la régulation des enzymes digestives et inhiberaient la prise de poids chez des larves de *Heliothis virescens* et *Helicoverpa zea* (Nachman and Pietrantonio, 2010). Chez la drosophile, la leucokinine jouerait également un rôle dans la prise alimentaire (Al-Anzi et al., 2010), et aurait une incidence directe sur la perception olfactive et gustative (López-Arias et al., 2011). Il pourrait avoir également un rôle dans les comportements stéréotypés précédant la mue imaginale (Kim et al., 2006).

Les leucokinines de différentes espèces d'insectes partagent des motifs communs (Radford et al., 2004). Le motif qui est nécessaire à leur activité est l'extrémité C terminale qui est constituée d'un pentapeptide Phe¹- Xaa₁²- Xaa₂³- Trp⁴- Gly⁵- NH₂ (avec Xaa₁² = His, Asn, Phe, Ser or Tyr; Xaa₂³ = Pro, Ser or Ala) (Nachman and Pietrantonio, 2010). Les récepteurs aux leucokinines sont généralement plus sensibles aux leucokinines de leur propre espèce mais ils sont activables aussi par leucokinines des autres espèces (Radford et al., 2004). Ces récepteurs sont des récepteurs membranaires couplés à des protéines G, qui activent des messagers secondaires. Chez la Drosophile, un seul récepteur a été

identifié, sur la base de son homologie avec un récepteur de tique (Holmes et al., 2000 in Nachman and Pietrantonio, 2010). Ce récepteur est exprimé dans les tubes de Malpighi, dans le tube digestif postérieur, dans le cerveau et dans les gonades mâles et femelles (Radford et al., 2002).

L'une des difficultés de travailler avec les leucokinines et les neuropeptides en général, est que leur durée de demi-vie est assez courte *in vivo*. Cette faible durée de vie est liée à l'activité des exo- et des endopeptidases présentes dans l'hémolymphe et le tube digestif des insectes. Nachman et al. (2002) ont identifié deux sites qui sont particulièrement sensibles à l'hydrolyse de ces enzymes. Ces observations ont conduit Nachman et collaborateurs à mettre au point des analogues peptidiques ou non peptidiques de kinine, dont la particularité est qu'ils possèdent un motif C-terminal analogue à la leucokinine et dont les sites attaqués par les enzymes sont protégés (Nachman et al., 2002; Taneja-Bageshwar et al., 2009). Ces kinines sont beaucoup plus stables que les neuropeptides endogènes et possèdent ainsi une activité diurétique et insecticide *in vivo* (Nachman et al., 2011).

Nous avons récemment montré que trois de ces analogues de leucokinine (1728, 1729 et 1490 : Kwon et al., 2016) ont une activité antiappétante et répulsive chez *Aedes aegypti*. Nous avons voulu établir si ces analogues avaient un effet similaire chez la drosophile. Pour ce faire, nous avons testé le comportement alimentaire de mouches adultes et testé les réponses de neurones gustatifs à des sucres et des composés amers, en présence de ces analogues.

2. Matériels et méthodes:

Les drosophiles testées étaient de la souche Canton S, élevées sur un milieu axénique et âgées de 3-5 jours. Les composés testés dans ce travail sont deux analogues de Kinine 1728 et 1729 (Figure 36) synthétisés dans le laboratoire du professeur Nachman. Les autres composés (saccharose, caféine, L-canavanine, TCC et erioglaucine) proviennent tous de Sigma Aldrich. Pour l'expérience de comportement, les solutions ont été mélangées avec de l'erioglaucine à 12.5 mg/100 ml. Pour les expériences d'électrophysiologie, les solutions contenaient de la tricholine citrate 30 mM.

Afin d'évaluer la consommation de solution sucrées par les mouches, nous avons testé des groupes de 20 mouches préparées comme précédemment, auxquelles on a proposé des choix binaires entre un capillaire contenant du saccharose 300 mM et l'analogue de kinine 1728.

Nous avons mesuré le nombre de potentiels d'actions sur des sensilles du proboscis L et i9. Les sensilles L possèdent 4 neurones gustatifs dont un seul répond au saccharose. Les sensilles i9 possèdent 2 neurones gustatifs, l'un qui est sensible aux sucres et l'autre à des substances amères.

3. Résultats

Lorsque les mouches ont le choix entre deux solutions de saccharose 100 mM dont l'une contient l'analogue de kinine 1728, nous observons une différence significative de consommation pour la concentration la plus élevée (1 mM) (Figure 40).

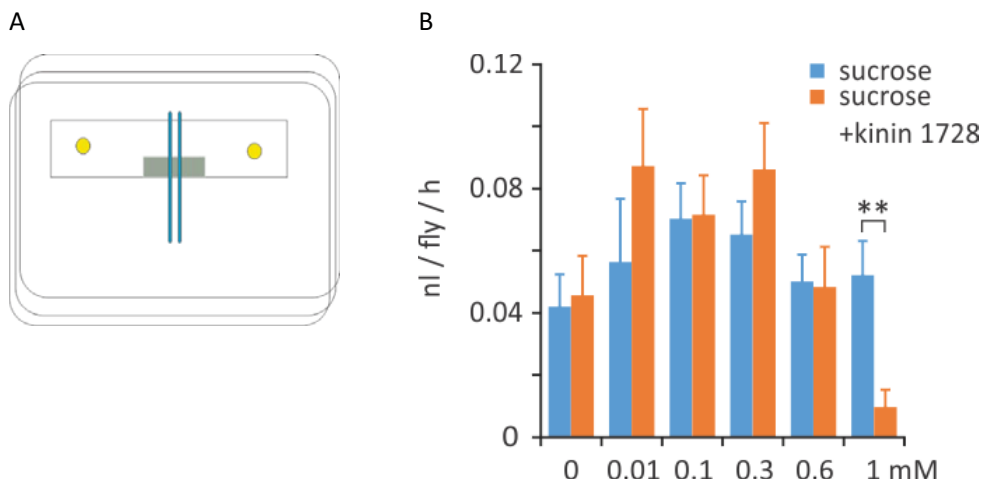


Figure 40 : Effet inhibiteur de l'analogue 1728 sur la consommation de saccharose chez la Drosophile

(A) Schéma de la disposition des capillaires utilisés. Deux capillaires de 5 μ l sont remplis d'une solution (bleue) et disposés sur une lame de verre de microscopie au fond d'une boîte en plastique transparent. Au début de l'expérience, 20 mouches sont introduites. Chaque boîte est alors scannée puis placée verticalement dans une enceinte à 25°C à l'obscurité. Au bout de 2 h, les boîtes sont scannées à nouveau. Chaque capillaire contient 5 μ l de saccharose 100 mM ; un des capillaires contient également l'analogue de kinine 1728 à 0, 0.01, 0.1, 0.3, 0.6 et 1 mM. (B) Consommation observée de chaque capillaire. On note une inhibition significative de la consommation avec 1mM de 1728 (test Wilcoxon Matched Pairs p value 0.005062). Chaque barre correspond à la moyenne de n=10 observations (+ SEM).

Cette inhibition de la consommation peut être liée à plusieurs facteurs : (i) la substance est toxique et la réduction d'alimentation est due à un effet post-ingestif, (ii) l'analogue de kinine inhibe la perception du sucre et (iii) l'analogue de kinine est détecté comme un stimulus amer. Nous avons écarté le premier mécanisme en considérant que la durée d'observation était trop courte pour qu'un tel mécanisme ait un rôle important sur les résultats. Afin d'estimer si l'analogue de kinine avait un effet sur la détection du sucre ou si il était détecté comme un stimulus amer, nous avons stimulé des sensilles L5 (qui contiennent 4 cellules gustatives mais ne sont pas sensibles à l'amer) et des sensilles I9 (qui contiennent 2 cellules, sensibles respectivement au sucre et à des substances amères).

Dans un premier temps, nous nous sommes demandés si l'analogue de kinine avait un effet inhibiteur sur la détection de sucre et si l'exposition à cet analogue induisait un effet inhibiteur prolongé. Nous avons donc réalisé une première série d'enregistrements sur des sensilles L, en stimulant les sensilles avec une solution de sucre (saccharose 100 mM), une solution de sucre

mélangée à un analogue de kinine (1728 ou 1729 à 1 mM), suivi par une stimulation de sucre seul afin d'évaluer un effet retard. Les résultats de cette expérience (Figure 41) montrent clairement que les deux analogues de kinine inhibent la détection du sucre à la concentration de 1 mM. L'analogue 1728 est plus efficace que 1729. Enfin, la récupération de la sensibilité initiale est totale, ce qui suggère que l'exposition brève (2 s) n'a pas d'effet retard sur le fonctionnement des sensilles gustatives et leur capacité à répondre au saccharose.

Cette inhibition dépend de la concentration de l'analogue de kinine (Figure 42). Le décrochement apparaît entre 0.1 mM et 0.3 mM. L'inhibition de la réponse est immédiate mais semble avoir un effet plus prononcé sur la partie tonique de la réponse dans les sensilles L.

Nous avons évalué si la présence d'un analogue de kinine avait un effet sur la détection d'une substance amère (la caféine) en présence de sucre (Figure 43). Avec l'analogue 1728, nous observons une faible réduction d'activité sur les sensilles i9 dans la gamme de concentrations testées (0.0001 à 0.1 mM). Cette réduction concerne surtout l'activité de la cellule sensible au sucre. Il semble par contre que l'analogue 1729 ait un effet prononcé sur les réponses à une dose de 0.1 mM dans cette expérience. Enfin, nous avons évalué l'effet de l'analogue 1728 à une concentration de 0.1 mM sur la réponse à 40 mM de L-canavanine (Figure 44). Aucune réduction de l'activité n'est observée.

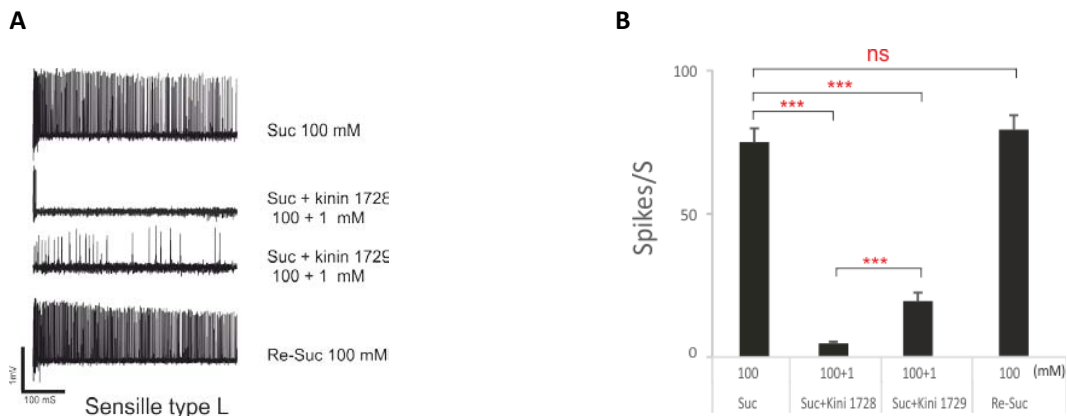


Figure 41 : Inhibition de la réponse au sucre par les analogues de kinine 1728 1729 chez la drosophile

(A) Exemples d'enregistrements extracellulaires (2 s) de sensilles L5 et L6 stimulées (du haut vers le bas) avec du saccharose 100 mM (Suc), Suc + un analogue de kinine 1728 (1 mM), Suc + analogue de kinine 1729 (1 mM) et enfin avec du saccharose 100 mM (Re-Suc). (B) Moyenne (\pm SEM) du nombre potentiels d'action détectés pendant la 1^{ère} seconde d'enregistrement ($n= 8-18$ sensilles). Les astérisques indiquent les différences significatives en effectuant un test de Mann-Whitney U (p value = 0.000017, 0.000071, 0.000428. Comparaison Suc>1728, Suc>1729, 1728>1729).

IV.- Modulation de la physiologie des récepteurs et détection des sucres

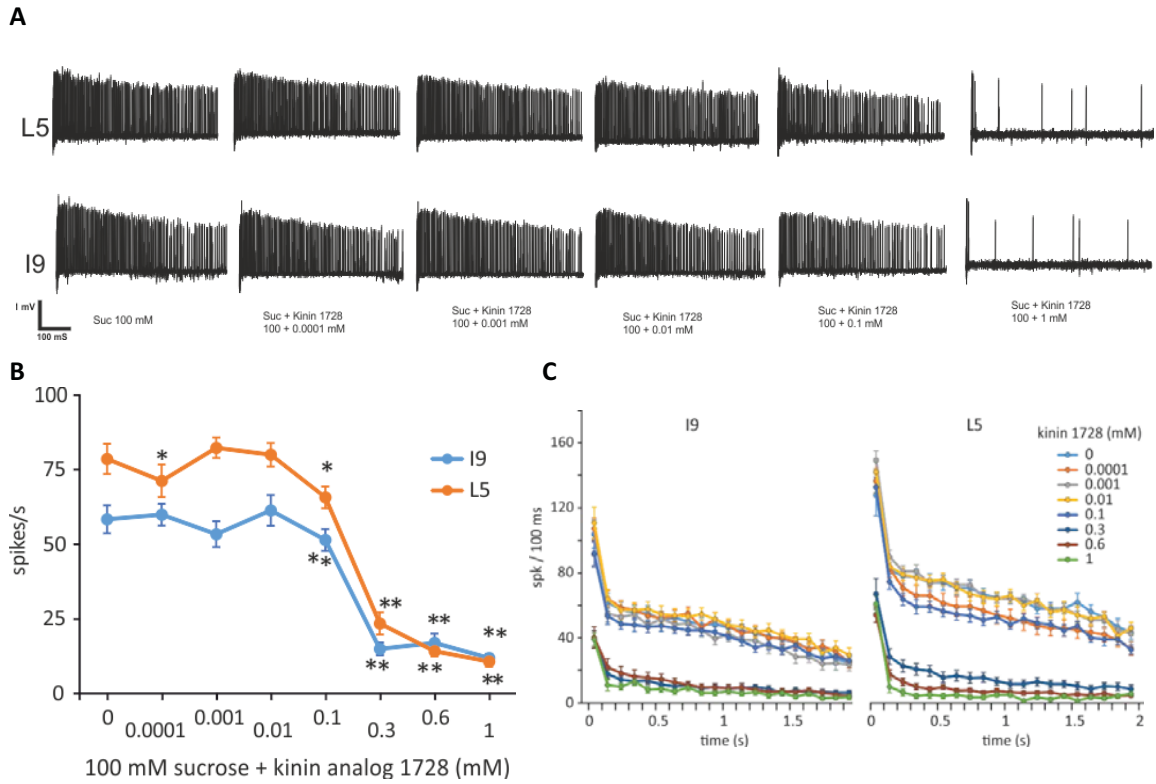


Figure 42 : Effet de la concentration de l'analog 1728 sur la réponse au sucre

(A) Exemples de réponses obtenues à du saccharose 100 mM en mélange avec de concentrations croissantes de l'analog de kinine 1728 sur des sensilles L5 et I9. (B) Moyenne (\pm SEM) du nombre de potentiels d'action détectés pendant la première seconde de stimulation. La réponse est significativement plus faible à partir de 0.1 mM et est presque nulle à 0.6 et 1 mM pour les deux sensilles. (Wilcoxon Matched Pairs ; $*=p<0.05$, $**=p<0.01$, $***=p<0.001$). (C) Réponses en fonction du temps (bin = 100 ms) et de la concentration de l'analog de kinine.

IV.- Modulation de la physiologie des récepteurs et détection des sucres

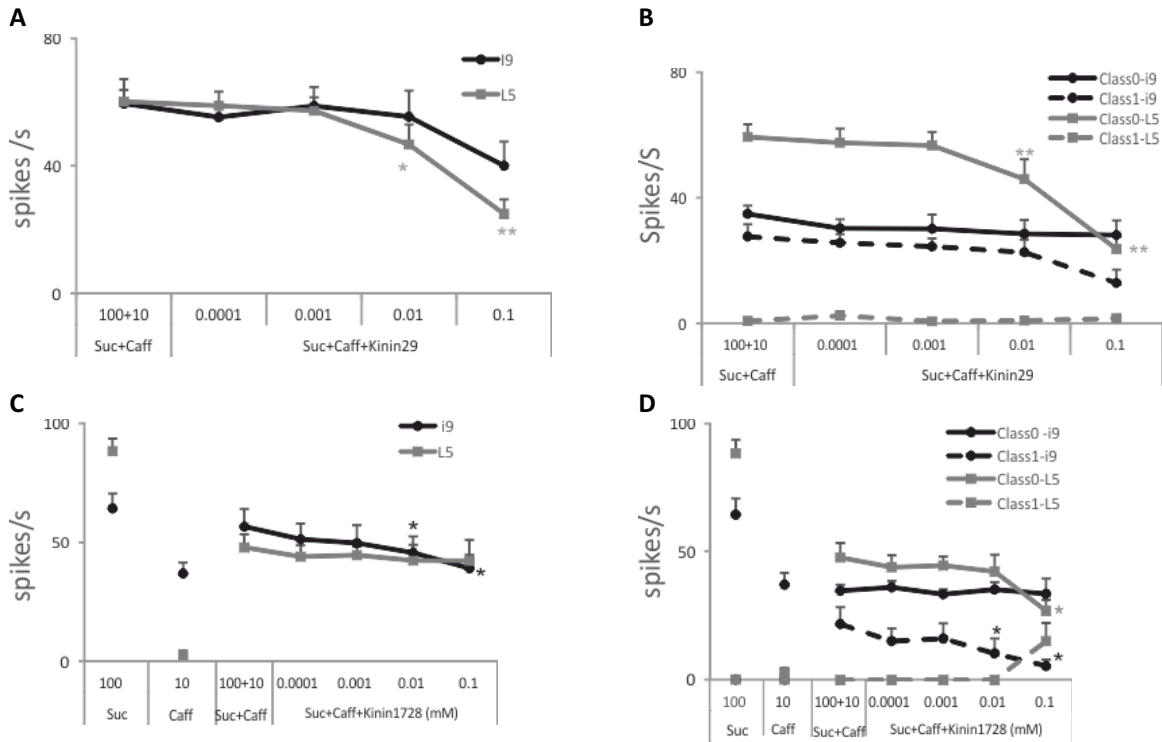


Figure 43 : Effets des analogues de kinine 1728 et 1729 sur les réponses gustatives à un mélange binaire de saccharose et de caféine

Réponse des sensilles L5 et I9 du proboscis avec du saccharose 100 mM, de la caféine 10 mM ou le mélange des deux auquel on a ajouté des concentrations croissantes de l'analogue 1728 ou 1729. (A-C) Nous avons séparé les potentiels d'action en deux classes d'amplitude et de forme. La classe 0 est activée en présence de sucre. La classe 1 est activée en présence de caféine. Ce sont les cellules de classe 0 qui sont affectées par la présence de l'analogue de kinine 1729. L'analogue 1728 influence l'activité de la classe 0 de I5 à la concentration de 0.1 mM et il diminue l'activité de la class 1 pour la sensille i9 aux concentrations 0.01 et 0.1 mM. Les astérisques indiquent la significativité. (B-D) Moyenne du nombre de potentiels d'action pendant 1s. L'analogue 1729 influence sur la sensille I5. L'analogue 1728 réduit la réponse de la sensille I9. n=6-10.

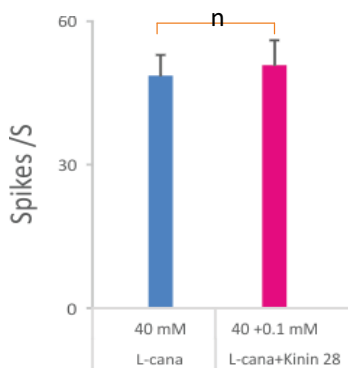


Figure 44: Réponse à la L-canavanine en présence de l'analogue 1728

L'analogue 1728 à 0.1 mM n'influence pas la réponse à la L-canavanine 40 mM dans les sensilles S5. en stimulant la sensille S5 du proboscis (test Wilcoxon Matched pairs et p value 0.892738). n=5

4. Discussion

Les observations que nous avons menées sur la Drosophile avec les analogues de kinine 1728 et 1729 montrent que ces analogues sont actifs sur cet insecte en réduisant la consommation de sucres et en inhibant la réponse des neurones sensibles aux sucres. Cette inhibition est très rapide et n'induit apparemment pas d'effets résiduels. Lorsque le sucre est en mélange avec une substance amère comme la caféine, l'inhibition porte principalement sur l'activité de la cellule répondant au sucre. Enfin, nous n'avons pas observé de diminution de la réponse à la L-canavanine en présence de l'analogue 1728.

Ces résultats sont très similaires à ceux que nous avons décrits sur le moustique ([Kwon et al., 2016](#)). L'inhibition observée sur la drosophile semble plus importante que chez le moustique. Il faut remarquer que les doses d'analogue utilisées dans notre essai sont très élevées (0.1 à 1 mM), surtout si on compare ces doses à celles qui sont mentionnées dans la littérature pour l'effet diurétique de la leucokinine (0.1 à 1 nM: [Taghert and Veenstra, 2003](#)). D'autres expériences sont certainement nécessaires pour vérifier que dans le cas de la drosophile, le récepteur LKR est bien la cible de ces analogues. Si cela était le cas, il est tentant de faire un parallèle entre l'activité de la kinine sur les cellules étoilées où l'activation du récepteur induit une augmentation du calcium intracellulaire qui ouvre des canaux Cl⁻ avec les observations de physiologistes qui ont montré que l'augmentation de calcium dans des cellules olfactives de vertébrés ([Delgado et al., 2016](#)) ou d'insectes ([Pézier et al., 2010](#)) ouvre également des canaux Cl⁻.

D. Détection de L- et D-arabinose [article 6]

1. Introduction

Dans tout ce qui précède, nous avons presque exclusivement considéré la détection de substances aversives, sans aborder la perception des sucres ni l'apprentissage. Nous avons été sollicités par le Dr Kausik Si (Stowers Institute for Medical Research, Kansas City) qui nous a demandé de faire des observations électrophysiologiques et comportementales sur la détection de l'arabinose. Cette recherche est partie de l'observation étonnante faite dans le laboratoire partenaire des effets différents de deux isomères d'un sucre non-nutritif, le L- et D arabinose. Alors que le D-arabinose est préféré par les mouches, lorsqu'une odeur est présente au moment de l'exposition au sucre durant 5 minutes, cette odeur est mieux mémorisée 24 h après l'exposition lorsqu'elle a été associée au L-arabinose. Afin de permettre de répondre à des questions spécifiques concernant les modalités de détection de l'arabinose et les comportements alimentaires associés, nous avons conduit des observations électrophysiologiques sur des sensilles du proboscis et des tarsi de drosophiles adultes. Nous avons aussi encadré une étudiante M1 en stage pendant 2 mois et demi, Palak Rawat (Amity University, Madhya Pradesh, Gwalior, Inde), qui a réalisé des observations comportementales du comportement alimentaire de drosophiles en présence de ces deux isomères.

2. Matériel et méthodes

Tous les tests ont été réalisés sur des drosophiles Canton S. Les molécules testées L- et D-arabinose provenaient de Sigma Aldrich. Nous avons réalisé des enregistrements électrophysiologiques comme précédemment décrit. Pour les observations comportementales, nous avons utilisé une chambre permettant d'enregistrer l'activité alimentaire des mouches au cours du temps (Figure 45).

3. Résultats

Les mouches ayant jeûné 22 h ont été mises en présence avec 5 capillaires de 5 μ l contenant du L- ou D-arabinose à 0, 1, 10, 100 et 1000 mM. La consommation de D-arabinose est immédiate mais s'arrête au bout de 20 minutes (Figure 46). La consommation de L-arabinose est moindre et elle s'étale sur 40 min pour la concentration la plus élevée.

IV.- Modulation de la physiologie des récepteurs et détection de sucres

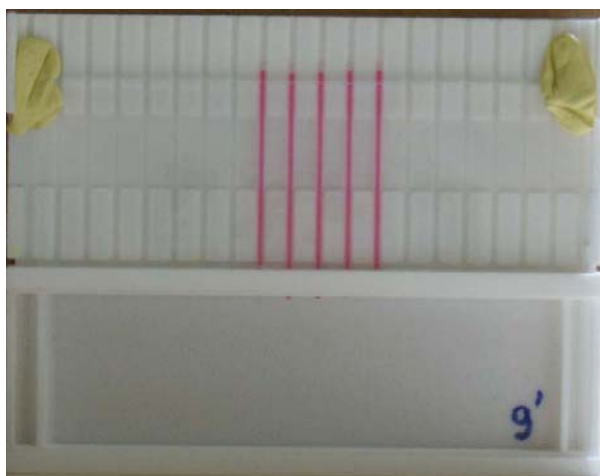
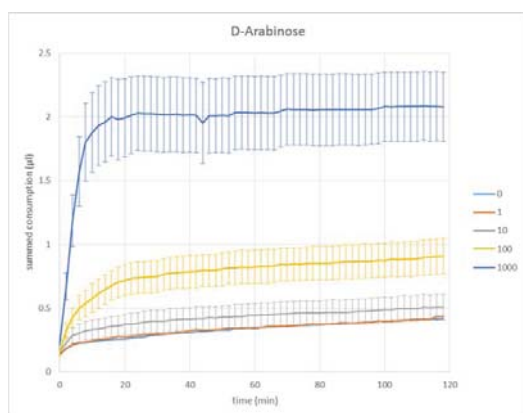


Figure 45: Dispositif utilisé pour les tests de comportement MultiCAFE

Dans ce dispositif, 20 mouches sont insérées dans une loquette fermée par une lame de microscope. Dans la partie supérieure de la loquette, des orifices sont ménagés pour laisser passer des capillaires de verre (sur la photo : 5) remplis d'une solution colorée à l'aide d'un colorant alimentaire. Ce dispositif est placé dans une boîte de type Tupperware, juste en dessous d'une webcam (Logitech C920) qui permet de capturer des images à intervalles de temps réguliers et donc de suivre au cours du temps la consommation des mouches.

A



B

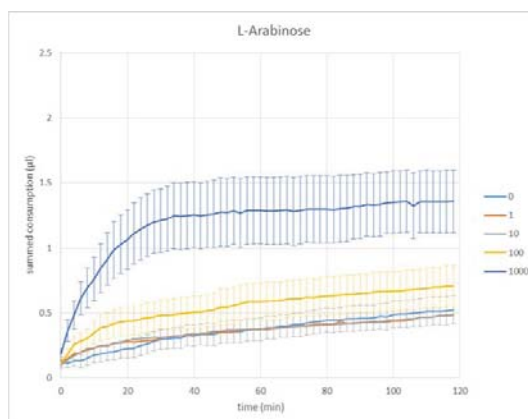


Figure 46 : Cinétique de la consommation de D- et L-arabinose dans un dispositif multiCAFE

Evolution temporelle de la consommation de D-arabinose (A) et de L arabinose (B) pendant 2 h, mesurée avec un pas de temps de 1 min. Il n'y a presque pas de consommation pour les concentrations moins de 1 M. Les mouches ($n= 20$ par boîte) ont jeûné 20 h en présence d'un papier filtre humidifié puis sont transférées dans le dispositif de multiCAFE en présence de 5 capillaires contenant une série de concentrations d'arabinose (0-1-10-100-1000 mM). Le graphe représente la moyenne de la consommation au cours du temps (\pm SEM). $n=12$ boîtes.

Nous avons ensuite évalué la réponse de sensilles gustatives du proboscis et des tarsi (Figure 47). Sur le proboscis, nous avons mesuré les réponses de la sensille L7. Sur les tarsi, nous avons enregistré l'activité de la sensille F5S. Les sucres ont été présentés aux concentrations de 0, 1, 10, 100 et 1000 mM. La réponse obtenue de la sensille L7 montre une différence entre les deux formes à la concentration la plus élevée (test Mann-Whitney U et $p= 0.000043$). Sur les tarsi, une différence significative est observée à partir de 1 mM (test Mann-Whitney U $p= 0.031043$, 0.021413 , 0.000000 , 0.000000 pour les concentrations 1-10-100-1000 mM respectivement) ?

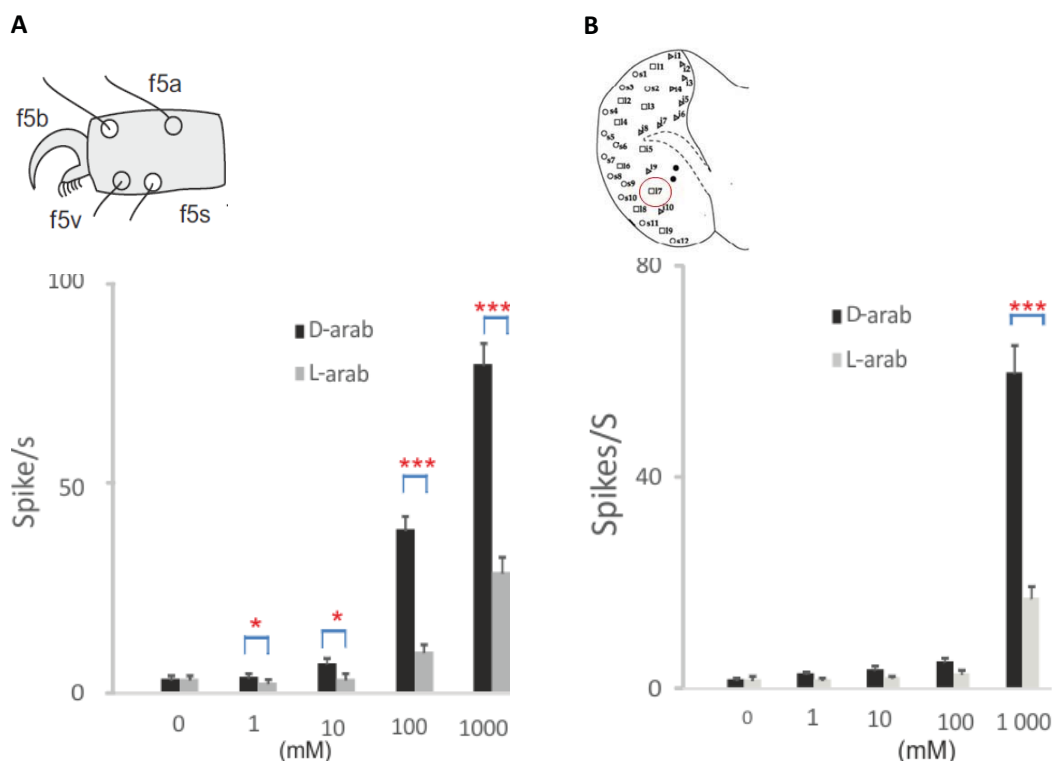


Figure 47: Réponses électrophysiologiques au L- et D-arabinose sur une sensille du proboscis et des tarse

(A) Moyenne des réponses de la sensille f5s à différentes concentrations de D- et L-arabinose (barres noires et grises, respectivement). (B) Réponses de la sensille L7 du proboscis au D- et L-arabinose. Les astérisques indiquent le niveau de significativité.

4. Discussion

Nos observations comportementales et électrophysiologiques montrent que les mouches préfèrent la forme D par rapport à la forme L de l'arabinose. Elles consomment et détectent la forme D plus rapidement que la forme L de cette molécule, mais elles cessent d'en ingérer en moins de 20 minutes. Le D-arabinose est très sucré mais pas nutritif au contraire du D-sorbitol qui est peu sucré et permet la survie des mouches (Fujita and Tanimura, 2011). L'arabinose est non seulement détecté par les sensilles périphérique comme nous l'avons montré ici, mais aussi par des neurones du pharynx (LeDue et al., 2015) grâce à *Gr43a*. Ce récepteur *Gr43a* est également exprimé dans les neurones de cerveau et joue un rôle dans la modulation du comportement alimentaire (Miyamoto et al., 2012). Les résultats que nous avons obtenus sont donc étonnants et suggèrent que l'évaluation des propriétés nutritives des sucres est très rapide.

V. Conclusions générales et perspectives

Il est important de comprendre les interactions insectes – plantes et en particulier les mécanismes qui permettent aux herbivores d'éviter ou consommer telle ou telle nourriture. La perception chimique (gustative ou olfactive) chez les insectes en général et chez la drosophile est cruciale pour bien comprendre les mécanismes de ces interactions. En particulier, on connaît bien maintenant plusieurs aspects essentiels du fonctionnement des systèmes chimiosensoriels des insectes :

- Un grand nombre de récepteurs olfactifs et gustatifs a été identifié chez la Drosophile (Clyne et al., 1999; Scott et al., 2001) et l'expression de ces récepteurs dans les différentes cellules gustative a été caractérisée (Weiss et al., 2011).
- Le rôle de certains de ces récepteurs a été caractérisé comme Gr66a et Gr93a (Lee et al., 2009) et *Or22a* and *Or47a* (Dobritsa et al., 2003). Dans ce contexte, l'expression de Gr66a a permis de définir que ce gène est nécessaire à la détection de la L-canavanine chez la drosophile, ce qui est similaire à ce que Lee et al. (2012) ont démontré et complètement différent de ce qui a été trouvé par Mitri et al. (2009) concernant DmXR.
- Bien que l'ensemble des récepteurs olfactifs et gustatifs soient généralement considérés comme des récepteurs ionotropiques (Amrein and Thorne, 2005; Wicher et al., 2008), nous avons contribué à démontrer que les récepteurs Gr66a et Gr8a et Gr98a qui sont indispensables à la détection de la L-canavanine ont besoin d'une liaison avec la protéine G α dans la voie de la transduction (Devambez et al., 2013).

Nous avons également abordé la détection de substances aversives en mélange. D'une part, nous avons participé à l'étude de l'inhibition de la détection des sucres par des substances amères, en collaboration avec les membres de notre équipe. Ce travail nous a montré que les neurones d'une modalité pouvaient être sensibles à des molécules d'une autre modalité. Ces interactions directes s'ajoutent d'autres types de modulations qui peuvent prendre place à la périphérie, par exemple des interactions épaptiques entre cellules sensorielles olfactives (Su et al., 2012), interactions directes que nous n'avons pas pu mettre en évidence dans le gustatif.

Nous nous sommes demandé ensuite si ces interactions entre l'amer et le sucré s'exerçaient dans les deux sens, et donc plus spécifiquement si les sucres pouvaient inhiber la détection de certaines substances amères. Nous avons trouvé que certains sucres présentés seuls ou en association avec un autre sucre, pouvaient diminuer la détection de substances amères. C'est le cas par exemple du saccharose / caféine ou du tréhalose / berberine. Des interactions similaires avaient été décrites chez *M. sexta* (Cocco and Glendinning, 2012). Afin de déterminer si ces modulations étaient dues à une inhibition latérale ou à des propriétés intrinsèques des neurones sensibles aux

substances amères, nous avons évalué l'effet des sucres sur la détection de substances amères chez des mouches dont les neurones sensibles aux sucres avaient été supprimés.

Ensuite, nous avons abordé la modulation de la détection des sucres sous l'influence d'un neuropeptide, en substituant au neuropeptide naturel des analogues capables d'activer les récepteurs natifs mais possédant une durée de vie plus longue que le neuropeptide naturel. Ces composés de synthèse nous ont permis de montrer que les neurones gustatifs exposés à ces analogues de kinine chez *A. aegypti* et chez la drosophile, perdent temporairement leur sensibilité au sucre mais apparemment pas aux substances amères.

Dans une dernière étape de notre travail, nous avons contribué à montrer que la drosophile est capable de détecter les formes D- et L- de l'arabinose et que la forme D- est beaucoup mieux détectée que la forme L- à la fois sur le proboscis et sur les tarsi. Cette différence de perception est étonnante car contrairement à ce qui est attendu, une odeur associée est mieux mémorisée lorsqu'elle est associée au L-arabinose qui est pourtant moins consommé et perçu avec moins d'intensité que le D-arabinose.

Nos résultats montrent pour la première fois chez la drosophile que certains sucres peuvent inhiber la détection de molécules amères, ce qui pourrait passer par une inhibition compétitive des récepteurs gustatifs de l'amer. Nous avons également observé que le mélange de deux molécules sucrées peut renforcer cette inhibition. Ces observations sont difficiles à expliquer avec les données actuellement disponibles et en particulier, il est difficile de comprendre comment et à quel niveau (moléculaire, métabolique, cellulaire) se place cette interaction?

Les résultats que nous avons obtenus avec les analogues de kinine chez *A.aegypti* et chez la drosophile montrent que ces analogues peuvent moduler la détection du saccharose directement au niveau des cellules gustatives. Ces effets suggèrent que de tels analogues peuvent être utiles pour étudier et identifier la modulation des récepteurs chimiosensoriels et *in fine*, des comportements. Là encore, la question des mécanismes impliqués reste complètement ouverte?

Dans la dernière partie de notre travail, nous avons comparé la détection de deux formes D et L de l'arabinose. Nos résultats démontrent que la détection de ces formes n'est pas identique à la fois sur le proboscis et sur les tarsi, mais aussi que les courbes doses-réponse obtenues pour ces deux formes ne sont apparemment pas superposables. Est-ce qu'on peut observer le même phénomène avec d'autres isomères de sucres? Et est-ce que les drosophiles sont capables de discriminer ces molécules comme des entités différentes ?

Afin d'aller plus loin et de répondre à une partie de ces questions, il faudrait peut-être utiliser d'autres approches et techniques. En particulier, la technique d'enregistrement que nous avons

utilisée limite la fenêtre d'observation du fonctionnement des neurones au temps pendant lequel l'électrode de stimulation est en contact avec les neurones gustatifs. Il serait intéressant de découpler la stimulation et l'enregistrement afin de pouvoir observer l'activité nerveuse avant et après la stimulation afin de pouvoir évaluer si les interactions inhibitrices que nous avons enregistrées continuent après la stimulation. En effet, dans la thèse de [Makoto Hiroi \(2016\)](#) qui avait montré quelques enregistrements de ce type, il apparaissait un rebond de l'activité en réponse à une stimulation avec certaines substances amères. Une telle technique sera utile surtout dans l'étape de tester l'interaction sucre –amer ou amer-sucre pour permettre de bien montrer si cette interaction concerne les neurones gustatifs ou directement les voies de transduction.

VI. Références

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VII. Les Annexes

A. Annexe 1: Liste des publications scientifiques

1. -< Articles>-

❖ *Gαo* Is Required for L-Canavanine Detection in *Drosophila*

Isabelle Devambez^{1,2,3}, Moutaz Ali Agha^{4,5,6}, Christian Mitri⁷, Joe' I Bockaert^{1,2,3}, Marie-Laure Parmentier^{1,2,3}, Frédéric Marion-Poll^{4,5,6}, Yves Grau^{1,2,3}, Laurent Soustelle^{1,2,3*}

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❖ Dual Mechanism for Bitter Avoidance in *Drosophila*

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❖ Leucokinin mimetic elicits aversive behavior in mosquito *Aedes aegypti* (L.) and inhibits the sugar taste neuro

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❖ **Immediate perception of a reward is distinct from the reward's long-term salience**

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[eLife](#) . 2016;10.7554/eLife.22283 (December 22, 2016)

❖ **Inhibition of bitter detection by sugars in *Drosophila melanogaster***

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sera soumis à [Chemical Senses](#)

❖ **Kinin analogs inhibition of sugar detection in *Drosophila melanogaster***

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(En preparation)

2. -< Review >-

❖ ***Drosophila* Bitter Taste(s)**

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[Frontiers in Integrative Neuroscience](#) | Volume 9 | Article 58 (November 2015).

Received: 07 October 2015 Accepted: 30 October 2015 Published: 25 November 2015.

3. -< Les Conférences >-

Présentation orale

The Perception of L-canavanine in the fly ; *Drosophila melanogaster*

Moutaz Ali Agha, Laurent Soustelle, Yves Grau and Frédéric Marion- Poll

« [6th Invertebrates Neurobiology Meeting](#) » 11-12 Juin 2015, Gif sur Yvette-France

Poster

➤ **Aversive effects of L-canavanine on *Drosophila***

Moutaz Ali Agha, Laurent Soustelle, Yves Grau and Frédéric Marion- Poll

« [25th Annual French Drosophila Conference](#) », 17-20 Octobre 2011, Lyon -France

➤ **The Gao protein is required for L-canavanine detection**

Moutaz Ali Agha, Laurent Soustelle, Yves Grau and Frédéric Marion- Poll

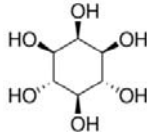
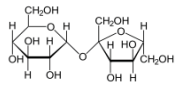
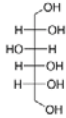
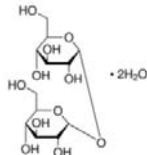
« [26th French Drosophila conférence](#) » 10-13 Septembre 2012, Clermont – Ferrand- France.

B. Annexe 2: Liste des sucres testés

Tableau 6 : Liste des sucres testés

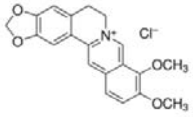
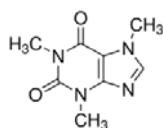
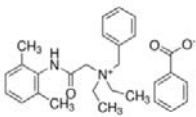
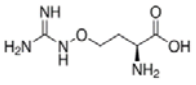
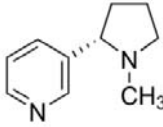
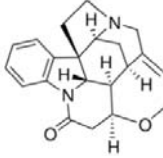
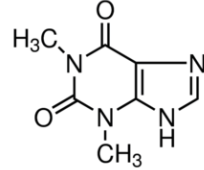
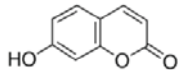
Sucre	Source	Pureté	Formule brute	Structure Chimique
D-Arabinose	Fluka	≥99%	C ₅ H ₁₀ O ₅	
L-Arabinose	Aldrich	98%	C ₅ H ₁₀ O ₅	
D-Fructose	Acros organics	90%	C ₆ H ₁₂ O ₆	
D-Galacturonic acid	Fluka	≥97%	C ₆ H ₁₀ O ₇ ·H ₂ O	
D-Glucose	Fluka	≥99.5%	C ₆ H ₁₂ O ₆	
Glycerol	Sigma	≥99%	C ₃ H ₈ O ₃	
D-Maltose	Aldrich	90%	C ₁₂ H ₂₂ O ₁₁ ·H ₂ O	
Maltotriose	Aldrich	95%	C ₁₈ H ₃₂ O ₁₆ ·xH ₂ O	
D-Melezitose	Sigma	≥99%	C ₁₈ H ₃₂ O ₁₆ ·xH ₂ O	

Annexe 2

myo-inositol	Sigma	≥99%	$C_6H_{12}O_6$	
D-Saccharose	Sigma	≥99.5%	$C_{12}H_{22}O_{11}$	
D-Sorbitol	Sigma	≥98%	$C_6H_{14}O_6$	
Trehalose D-	Sigma	≥99%	$C_{12}H_{22}O_{11} \cdot 2H_2O$	

C. Annexe 3: Liste des substances amères testées

Tableau 7: Liste des substances amères testées

Amer	Source	Pureté	Formule brute	Structure Chimique
Berberine chloride	Sigma	-	$C_{20}H_{18}ClNO_4$	
Caféine	Sigma	-	$C_8H_{10}N_4O_2$	
Denatonium benzoate	Fluka	≥99%	$C_{28}H_{34}N_2O_3$	
L-canavanine	Sigma	≥98%	$C_5H_{12}N_4O_3$	
Nicotine	Sigma	≥99%	$C_{10}H_{14}N_2$	
Strychnine	Sigma	-	$C_{21}H_{22}N_2O_2 \cdot HNO_2$	
Théophylline	Sigma	99%	$C_7H_8N_4O_2$	
Umbelliferone	Aldrich	99%	$C_9H_6O_3$	

D. Annexe 4: Liste des souches de mouches

Tableau 8 : Liste des souches utilisées

Souche	Origine
<i>Drosophila melanogaster</i>	
Canton S (CS)	Tanimura – Fukuoka, Japan
W ¹¹¹⁸	Scott – Berkeley, USA
w* ; Gr66a-Gal4 (II)	Laurent Soustelle - Montpellier, France
UAS-TETxLC	Laurent Soustelle – Montpellier, France
w; UAS-DmXR (III)	Yves Grau - Montpellier, France
Gr66a-Gal4 / CyO ; TM2 / TM6B	Tanimura – Fukuoka, Japan
w ; f06268 / CyO	Yves Grau - Montpellier, France
w ; df(2R)Ex7096 / CyO	Yves Grau - Montpellier, France
UAS-PTX / TM3, SB	Laurent Soustelle - Montpellier, France
w; Gr33a ¹	Craig Montell, California, USA
Gr5a-Gal4 / CyO ; TM2 / TM6B	Makoto Hiroi/ Scott, Berkeley, France
w; ΔGr66a ex83	Yves Grau- Montpellier, France
UAS-RNAi Gα0	Yves Grau- Montpellier, France
<i>Aedes aegypti</i>	Institut Pasteur - Paris, France

E. Annexe 5: Liste de référence des substances testées

Nom	Source	Pureté	CAS
D-Glucose anhydrous	Fluka	≥99.5%	492-62-6
D-Maltose monohydrate	Aldrich	90%	11-256-9
Maltotriose hydrate	Aldrich	95%	1109-28-0
D-Saccharose	Sigma	≥99.5%	57-50-1
Glycerol	Sigma	≥99%	56-81-5
D-Fructose	Acros organics	90%	57-48-7
D-Melezitose hydrate	Sigma	≥99%	207511-10-2
D-Trehalose dihydrate	Sigma	≥99%	6138-23-4
Myo-inositol	Sigma	≥99%	87-89-8
D-Galacturonic acid monohydrate	Fluka	≥97%	91510-62-2
D-Arabinose	Fluka	≥99%	10323-20-3
D-Sorbitol	Sigma	≥98%	50-70-4
Berberine Chloride	Sigma	-	633-66-9
Caffeine	Sigma -Aldrich	-	58-08-2
Denatonium benzoate	Fluka	≥99%	3734-33-6
Umbelliferone	Aldrich	99%	93-35-6
Theophylline, anhydrous	Sigma	>99%	58-55-9
L-canavanine	Sigma	≥98%	543-38-4
Nicotine hydrogen tartrate salt	Sigma	≥98%	65-31-6
Lobeline hydrochloride	Aldrich	98%	134-63-4
Strychnine	Sigma	-	57-24-9

**F. Annexe 6: Composition de milieu axénique utilisé
pour l'élevage des mouches (*Drosophila*)**

Pour préparer 10 litres de milieu

- 120g d'Agar
- 830g de levure
- 830g de gaude
- 69g de nipagine
- 500mL d'alcool

VIII. Les publications scientifiques

A. Article 1 : Gαo is required for L- canavanine detection in *Drosophila*

PLOS ONE. 2013 May 6, 8(5): e63484. [doi:10.1371/journal.pone.0063484](https://doi.org/10.1371/journal.pone.0063484)

Abstract

Taste is an essential sense for the survival of most organisms. In insects, taste is particularly important as it allows to detect and avoid ingesting many plant toxins, such as L-canavanine. We previously showed that L-canavanine is toxic for *Drosophila melanogaster* and that flies are able to detect this toxin in the food. L-canavanine is a ligand of DmXR, a variant G-protein coupled receptor (GPCR) belonging to the metabotropic glutamate receptor subfamily that is expressed in bitter-sensitive taste neurons of *Drosophila*. To transduce the signal intracellularly, GPCR activate heterotrimeric G proteins constituted of α , β and γ subunits. The aim of this study was to identify which G α protein was required for L-canavanine detection in *Drosophila*. By using a pharmacological approach, we first demonstrated that DmXR has the best coupling with G α_o protein subtype. Then, by using genetic, behavioral assays and electrophysiology, we found that G α_o47A is required in bitter-sensitive taste neurons for L-canavanine sensitivity. In conclusion, our study revealed that G α_o47A plays a crucial role in L-canavanine detection.

Gαo Is Required for L-Canavanine Detection in *Drosophila*

Isabelle Devambez^{1,2,3}, Moutaz Ali Agha^{4,5,6}, Christian Mitri⁷, Joël Bockaert^{1,2,3}, Marie-Laure Parmentier^{1,2,3}, Frédéric Marion-Poll^{4,5,6}, Yves Grau^{1,2,3}, Laurent Soustelle^{1,2,3*}

1 CNRS, UMR 5203, Institut de Génétique Fonctionnelle, Montpellier, France, **2** INSERM, U661, Montpellier, France, **3** Universités de Montpellier 1 & 2, UMR 5203, Montpellier, France, **4** INRA, UMR 1272, Physiologie de l'Insecte, Versailles, France, **5** Université Pierre et Marie Curie, UMR 1272 Physiologie de l'Insecte, Versailles, France, **6** AgroParisTech, Département Sciences de la Vie et Santé, Paris, France, **7** Institut Pasteur, URA3012 CNRS, Unité Génétique et Génomique des Insectes Vecteurs, Paris

Abstract

Taste is an essential sense for the survival of most organisms. In insects, taste is particularly important as it allows to detect and avoid ingesting many plant toxins, such as L-canavanine. We previously showed that L-canavanine is toxic for *Drosophila melanogaster* and that flies are able to detect this toxin in the food. L-canavanine is a ligand of DmXR, a variant G-protein coupled receptor (GPCR) belonging to the metabotropic glutamate receptor subfamily that is expressed in bitter-sensitive taste neurons of *Drosophila*. To transduce the signal intracellularly, GPCR activate heterotrimeric G proteins constituted of α , β and γ subunits. The aim of this study was to identify which G α protein was required for L-canavanine detection in *Drosophila*. By using a pharmacological approach, we first demonstrated that DmXR has the best coupling with G α_o protein subtype. Then, by using genetic, behavioral assays and electrophysiology, we found that G α_o47A is required in bitter-sensitive taste neurons for L-canavanine sensitivity. In conclusion, our study revealed that G α_o47A plays a crucial role in L-canavanine detection.

Citation: Devambez I, Ali Agha M, Mitri C, Bockaert J, Parmentier M-L, et al. (2013) G α_o Is Required for L-Canavanine Detection in *Drosophila*. PLoS ONE 8(5): e63484. doi:10.1371/journal.pone.0063484

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Competing Interests: Co-authors Marie-Laure Parmentier and Frédéric Marion-Poll are PLOS ONE Editorial Board members. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

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Introduction

Taste is an important chemosensory cue, which is crucial for the survival of any organisms as it prevents the ingestion of toxic compounds. Toxins often have a bitter taste, explaining why the activation of bitter-sensitive taste neurons is generally associated with a rejection behavior. This reaction to bitter molecules is found in vertebrates but also in the fruit fly *Drosophila*, which react similarly to human for various tastants [1].

As a defense mechanism against predators, plants have developed toxins and antifeedants such as L-canavanine. The toxicity of L-canavanine is due to its structural similarities with L-arginine, leading to its incorporation into *de novo* synthesized proteins, making them not functional [2]. We previously showed that forced ingestion of L-canavanine is deleterious to *Drosophila melanogaster* and that this organism has the capacity to detect the presence of L-canavanine into the food, preventing its ingestion [3]. Thus, L-canavanine acts as a repellent molecule for fruit flies.

Drosophila taste neurons (also called gustatory receptor neurons, GRNs) are found in sensilla that are localized in the proboscis, legs, wings as well as the ovipositor [4]. Each sensillum houses two to four GRNs, which are dedicated to different taste modalities. Indeed, *Drosophila* gustatory system is able to detect sugars, bitter/toxic compounds, salts and water [4]. Recent studies have also shown that the *Drosophila* gustatory system is involved in pheromone detection and plays a role in courtship [5,6].

The first family of taste receptors identified in *Drosophila melanogaster* were members of the Gustatory Receptors (GRs) family that include 60 genes predicted to encode 68 proteins generated by alternative splicing. Most of them are expressed in bitter-sensitive GRNs [7]. In addition, most if not all bitter-sensitive GRNs express GR66a, which was originally described as a caffeine receptor [8]. Caffeine is repellent for *Drosophila* and its detection not only requires GR66a but also, at least, GR33a and GR93a as the mutation of one of these three GRs impaired caffeine detection [9]. Also, Lee and collaborators found that the detection of the synthetic repellent compound DEET required GR32a, GR33a, and GR66a and suggested that GRs may act in a heteromultimeric complex [10]. In addition, it was suggested that GR33a is an indispensable co-receptor for bitter compounds as GR33a mutant flies are impaired for the perception of most of them [9]. A similar situation was found for the detection of most sugars, where it was shown that GR64f is a co-receptor of GR5a and GR64a [11]. Because GRs are seven transmembrane proteins, it was originally thought that they were G-protein coupled receptors (GPCRs) [12,13]. However, GRs, like members of the related olfactory receptor (OR) family, have an inverted topology compared to GPCRs [14,15,16]. Recent studies have highlighted the repertoire diversity of taste receptors in *Drosophila*. Indeed, members of the degenerin/epithelial sodium/pickpocket (DEG/EnaC/ppk) channel family are involved in water and salt taste detection [17,18,19] and TRPA1, a member of the Transient

Receptor Potential (TRP) channel family, detects reactive electrophiles [20], such as allyl isothiocyanate, which gives a pungent taste to mustard and wasabi.

We have previously published that L-canavanine detection and associated behaviors rely on a GPCR called DmX [3]. The DmX receptor belongs to the metabotropic glutamate receptor (mGluR) family but it is not activated by glutamate due to conserved modifications within its ligand binding pocket [21]. We also found that L-canavanine binds and activates DmXR in HEK transfected cells [3]. However, a recent report has also shown that GR66a and GR8a, two members of the GR family, were involved in L-canavanine detection [22].

Canonical GPCR signaling relies on an intracellular heterotrimer of G proteins constituted of one G α , one G β and one G γ subunit. In its inactive state, the G α subunit is bound to GDP. Upon GPCR activation, GDP is replaced by GTP and subsequently GTP-bound G α and G β / γ subunits dissociate to activate downstream effectors [23]. Classically, mammalian G α proteins are divided into four subfamilies based on sequence similarities: G α_s , G $\alpha_{i/o}$, G $\alpha_{q/11}$ and G $\alpha_{12/13}$ [24]. The G α_s and G $\alpha_{i/o}$ subfamilies were named for their ability to stimulate and inhibit adenylyl cyclase isoforms, respectively. The G $\alpha_{q/11}$ subfamily is linked to the stimulation of phospholipase C β while the G $\alpha_{12/13}$ subfamily activates the small G protein Rho pathways [24].

Here to better understand the signaling pathway involved in L-canavanine detection in bitter-sensitive taste neurons, we focused on G proteins, asking if any G α is required for L-canavanine sensitivity. We first used a pharmacological approach to determine which G α protein has the best coupling to DmXR and found that DmXR can transduce the signal via G α_o subtype in HEK transfected cells. Then, we performed genetic and behavioral experiments and found that G α_o47A , the only G α_o member in the *Drosophila melanogaster* genome, is required in bitter-sensitive taste neurons for L-canavanine detection. Finally, by using an electrophysiological approach, we confirmed that blocking G α_o47A function led to a very strong reduction in L-canavanine sensitivity and has no other impact on the bitter taste neurons, as caffeine detection was normal.

Altogether, our data showed that G α_o47A is required for L-canavanine detection in bitter-sensitive taste neurons of *Drosophila*.

Materials and Methods

Cell culture, transfection and inositol phosphate (IP) assay

HEK 293 cells were cultured as described in [25] and transiently transfected by electroporation with either 14 μ g of carrier DNA (pRK), plasmid DNA containing HA-DmXR wild-type, plasmid DNA containing G α protein (2 μ g) (into pcDNA3.1, Invitrogen). Several G α proteins were used, including wild type (G α_{15} , G α_{16} , G α_q) or chimeric (G α_{q05} , G α_{q19} , G α_{qZ5}) proteins [26]. All these wild type and chimeric G α proteins are known to activate phospholipase C [26]. Determination of inositol phosphate (IP) accumulation in transfected cells was performed after labeling the cells overnight with [³H]myoinositol (23.4 Ci/mol) as described previously [27]. The stimulation was conducted for 30 min in a medium containing 10 mM LiCl and 10 mM L-canavanine. The basal IP formation was determined after 30-min incubation in the presence of 10 mM LiCl. Results are expressed as the amount of IP produced divided by the radioactivity present in the membranes. L-canavanine was purchased from Sigma (#c1625).

Fly stocks

CantonS flies were used as wild-type and w¹¹¹⁸ flies were used as a control for electrophysiological experiments. Gr66a-Gal4 line was a gift from H. Amrein (Texas A&M Health Science Center, College Station). UAS-RNAiG α_i65A (stock 28150) and UAS-RNAiG α_o47A (stock 19124) lines were obtained at the Vienna *Drosophila* RNAi Center (VDRC). UAS-G α_o^{GDP} carried a mutant form of G α_o (G203T mutation), which mimicked the GDP-bound state of G α_o protein [28]. This line was a gift from A. Tomlinson (Columbia University). The UAS-PTX line was a gift from G. Roman (University of Houston) [29].

PER/PPR assay

The proboscis extension reflex (PER) and the premature proboscis retraction (PPR) were examined as described in [3]. Briefly, adult flies were maintained on fresh medium and then starved on water-saturated cotton for 20 h. Flies were then immobilized by chilling them on ice and mounted ventral-side-up using myristic acid. Flies were allowed to recover for two hours in humid conditions. Before the assay, flies were satiated with water until no proboscis extension was elicited by water stimulation. Each fly was tested during 5 s by touching only the leg tarsi with either a 100 mM sucrose solution or 100 mM sucrose+40 mM L-canavanine solution. Six to eight batches of 40–60 flies were tested for each solution and each genotype. The occurrence of PER and PPR was determined during the assay. The percentage of PPR represents the number of flies that showed the PPR phenotype divided by the number of flies that have shown a PER. Unpaired Student t-tests were used to check for significant differences between the indicated pairs of data.

Two-choice feeding test

For each trial, between 80 to 100 adult flies (3- to 5-days old) were starved on water-saturated cotton for 24 h. Flies were then placed on a 60-well microtiter plate (#56243, Dutschern France) at 25°C during two hours in the dark. Wells contained 1% agarose with 0.15 mg/ml erioglaucine dye (blue) or 0.2 mg/ml sulforhodamine B dye (red) in the alternating wells. The sucrose concentrations were 5 and 1 mM in the blue and red solutions, respectively. After 2 h on the plates, the flies were frozen and the numbers of flies that were blue (NB), red (NR), or purple (NP) were determined on the basis of the colors of their abdomen. The preference index (PI) values for the blue solution were calculated according to the following equation: (NB+0.5NP)/(NB+NP+NR). A PI value of 1 or 0 indicates a complete preference or aversion, respectively. A PI value of 0.5 indicates no preference/aversion. In all the tests shown, the L-canavanine was added to the blue solution. Four independent trials were carried out for each condition. Unpaired Student t-tests were used to check for significant differences between the indicated pairs of data.

Electrophysiological recordings

For electrophysiological recordings, 4 days old flies were briefly numbed in ice and then restrained on their side on putty (UHU Patafix®), using fine strips of semi-transparent tape. A silver wire connected to the electrical ground was maintained close to their abdomen and a drop of electrocardiogram gel (Redux Gel, Parker Laboratories, Fairfield NJ, USA) was then deposited over it, thus providing an electrical reference and ensuring a minimal stress to the insect. The preparation was then left to rest about 30 min to 1 h before recordings occurred. The preparation was brought under a microscope (Leica MZ16), and properly oriented so that the S6 sensillum on the proboscis was accessible to stimulation (see

map from [30]). As for stimulation, we used borosilicate glass capillaries (tip size about 10 μm; Harvard Apparatus LTD, Edenbridge, UK), filled with the stimulus solution and 1 mM KCl, which served as an electrolyte. This electrode was connected to a taste amplifier (TastePROBE DT-02, [31]), which triggered upon contact a 2 seconds recording bout with a 16 bits data acquisition board (DT9803, Data Translation, USA) sampling data at 10 kHz, under the control of a custom program (dbWave; [32]). Data were further amplified (×500) and filtered (10–2800 Hz) with a CyberAmp 320 amplifier (Axon Instruments, USA). The number of spikes occurring during each recording was detected using dbWave and exported to a spreadsheet for further analysis. Unpaired Student t-tests were used to check for significant differences between the indicated pairs of data.

Results

The G-protein coupled receptor DmX is coupled to Gα_{i/o} protein subtype *in vitro*

DmXR belongs to the metabotropic glutamate receptors (mGluRs) subfamily, which includes eight members in vertebrates. mGluR1-5 are positively coupled to phospholipase C (PLC) via Gα_q, while mGluR2,3,4,6,7 and 8 are negatively coupled to adenylyl cyclase via Gα protein of i/o subtype [33]. The intracellular domains of mGluRs have been extensively studied and are responsible for the specificity of coupling to specific G-proteins, especially the second intracellular loop [34,35]. Hence, all Gα_{i/o} coupled mGluRs share identical residues at different positions of the intracellular loops, and these residues are different in mGluR1 and 5 (Fig. 1). To get a hint on the G-protein-coupling specificity of DmXR, we first analyzed its intracellular loop sequences and found that DmXR share the conserved residues of Gα_{i/o}-coupled mGluRs instead of those of mGluR1 and 5 (Fig. 1). Thus, DmXR may be coupled to Gα_i or Gα_o, or both.

The ability of individual Gα protein to discriminate specific GPCRs is linked to the presence of specific residues localized within the C-terminal region of the Gα subunits [26]. Taking advantage of this observation, chimeric Gα proteins have been made by replacing the 5 to 9 C-terminal residues of Gα_q protein by the corresponding residues of Gα_{i/o} or Gα_z proteins (the latter being a divergent member of the Gα_{i/o} family). These proteins are denoted Gα_{qi9}, Gα_{qo5} and Gα_{qz5} respectively. Importantly, the coupling specificity of these chimeric Gα proteins towards GPCRs is conserved [26], *i.e.* Gα_{qi9} is activated by Gα_i coupled receptors. Note that these chimeric G-proteins activate PLC, like Gα_q, instead of inhibiting adenylyl cyclase [26]. Hence, these chimeric proteins, as well as other wild-type Gα proteins that activate PLC (Gα₁₅, Gα₁₆ and Gα_q) allows to characterize the G-protein

coupling profile of DmXR by using a single *in vitro* assay: the measure of ligand-induced inositol phosphate (IP) production.

Human embryonic kidney (HEK) cells were co-transfected with expression vectors carrying DmXR without or with one Gα protein subtype, including wild type (Gα₁₅, Gα₁₆ and Gα_q) or chimeric (Gα_{qi9}, Gα_{qo5} and Gα_{qz5}) proteins. We then measured the IP production in presence or absence of L-canavanine, the known ligand of DmXR [3]. Data shown in Fig. 2 indicate that the strongest L-canavanine-induced DmXR activation was found when HEK cells co-expressed Gα_{qo5}. A weakest, but statistically significant, IP production was observed with HEK cells co-expressing DmXR and Gα_{qi9} (Fig. 2). As expected, we detected L-canavanine-induced DmXR activation by using the Gα₁₅ protein, which is known to couple to most types of GPCRs [36,37]. In contrast, no L-canavanine-induced DmXR activation was observed when HEK cells were co-transfected with Gα₁₆, Gα_q or Gα_{qz5} (Fig. 2), indicating that DmXR was not coupled to such types of Gα proteins, at least in HEK cells. Thus, we conclude that DmXR is a GPCR that couples to Gα_{i/o} proteins.

Gαo47A, but not Gαi65a, is required in bitter-sensitive neurons for L-canavanine-induced premature proboscis retraction

In the *Drosophila melanogaster* genome, two genes encoding Gα_{i/o} subtypes of G proteins are present: Gαi65A (CG10060) and Gαo47A (CG2204). In order to determine which Gα protein is required for L-canavanine detection *in vivo*, we used flies expressing specific RNAi against each of these two G proteins, specifically in bitter-sensitive taste neurons and performed behavioral analyses. One paradigm to study taste in flies is the proboscis extension reflex (PER) assay [38]. During this test, the stimulation of leg tarsi with a sucrose solution induces an extension of the proboscis, which is maintained several seconds. When a deterrent compound is added to a sucrose solution, the reflex is blocked and flies do not extend their proboscis. This inhibitory effect on sucrose-induced proboscis extension reflex was observed for most deterrent compounds such as caffeine, strychnine and quinine but not for L-canavanine [3,38]. Indeed, we previously found that the stimulation of leg tarsi with a L-canavanine and sucrose mixed solution induced a premature proboscis retraction (PPR), *i.e.* the flies extended their proboscis but retracted it almost immediately [3]. By using the Gr66a-Gal4 driver, which targets all bitter-sensitive taste neurons, we expressed RNAi construct against Gαi65A or Gαo47A and analyzed PPR phenotypes in presence or not of L-canavanine. Data shown in Fig. 3 indicate that all genotypes tested had a very low percentage of PPR when a sucrose solution was used for leg tarsi stimulation, indicating that flies detected sucrose correctly and maintained their proboscis extend-

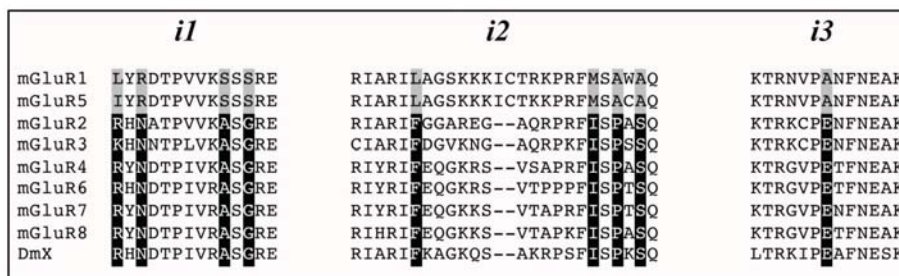


Figure 1. Sequence alignment of the intracellular loops of mGluRs and DmXR. *i1*, *i2*, and *i3* correspond to the first, second, and third intracellular loops of mGluRs and DmXR, respectively. Residues conserved in mGluRs coupled to phospholipase C (mGluR1 and 5) are boxed in grey, and the corresponding residues in most adenylyl cyclase coupled mGluRs (mGluR2, 3, 4, 6, 7 and 8) and DmXR are boxed in black, respectively. doi:10.1371/journal.pone.0063484.g001

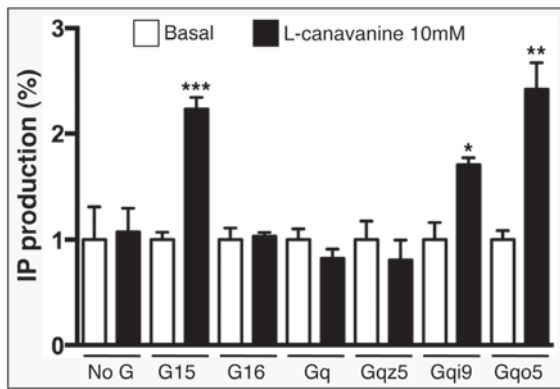


Figure 2. The GPCR DmX has the best coupling with G α protein subtype in HEK transfected cells. L-canavanine induced-inositol phosphate (IP) production was measured from HEK cells co-expressing the DmX receptor and the indicated G α protein. As a control, we used HEK cells transfected with DmXR expression vector alone (called 'No G'). Basal and 10 mM L-canavanine were used for all stimulations, indicated by white and black bars, respectively. IP stimulation was calculated relatively to IP production in basal conditions. HEK cells co-expressing DmXR and G α_{15} , G α_{q19} or G α_{qo5} produced IP after L-canavanine stimulation, indicating that these G α proteins can efficiently couple to DmXR, the best coupling being observed with G α_{qo5} . No such effect was observed with HEK cells co-expressing DmXR and G α_{16} , G α_q or G α_{qz5} . Experiments done with G α_{15} could be considered as a positive control because G α_{15} protein is known to couple with most GPCRs. Data are means \pm SEM from triplicate experiments. IP production was compared with basal activity using Unpaired Student's *t* test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). doi:10.1371/journal.pone.0063484.g002

ed. In contrast, Gr66a-Gal4/+, UAS-RNAiG α_{o47A} /+, UAS-RNAiG α_{i65A} /+ control flies and Gr66a-Gal4/++;UAS-RNAi-G α_{i65A} /+ flies presented a high percentage of PPR when a L-canavanine and sucrose mixed solution was used (Fig. 3). This revealed that these flies detected L-canavanine and retracted prematurely their proboscis, excluding a role of G α_{i65A} in the signaling pathway linked with L-canavanine detection. On the contrary, a similar low percentage of PPR was obtained with the sucrose and the L-canavanine/sucrose mixed solution on Gr66a-Gal4/++;UAS-RNAiG α_{o47A} /+ flies. This experiment demonstrates that the down-regulation of G α_{o47A} in bitter-sensitive taste neurons impaired L-canavanine sensitivity (Fig. 3). These data strongly suggest that G α_{o47A} , but not G α_{i65A} , plays a role in L-canavanine detection *in vivo*.

L-canavanine detection is impaired in flies expressing G α_{o47A} RNAi or a dominant negative G α (G α^{GDP}) in bitter-sensitive taste neurons

In order to confirm these data, we used another behavioral assay: the two choice feeding test, which measures the consumption of sucrose solutions colored by two food dyes of different colors (blue/red) offered simultaneously to flies. In this test, the blue solution contained more sucrose (5 mM) compared to the red one (1 mM), inducing an attraction of wild-type flies towards the blue solution as shown in Fig. 4 (wild-type in white bar). When L-canavanine (30 mM) was added to the blue sucrose solution, wild-type flies detected it and avoided eating the blue solution (Fig. 4, wild-type in black bar), consistently with the repulsive effect of L-canavanine. By using this test, we found that RNAi knock-down of G α_{o47A} in bitter-sensitive taste neurons impaired L-canavanine detection but had not effect on sucrose attraction (Fig. 4). Similar results were obtained with a G α mutant construct (Fig. 4), known

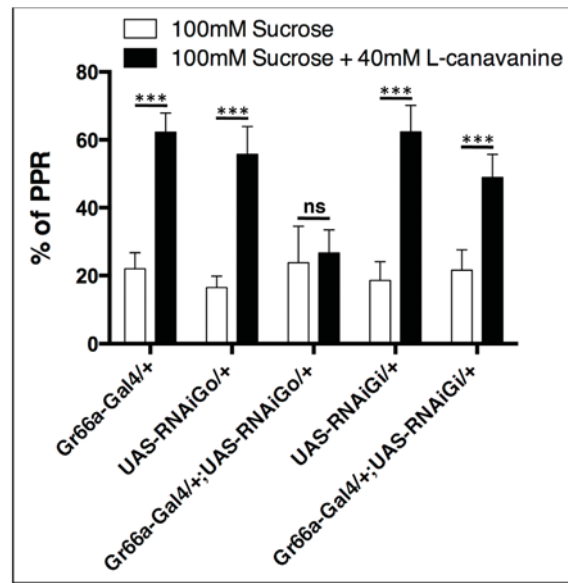


Figure 3. RNAi knockdown of G α_{o47A} in bitter-sensitive taste neurons impairs L-canavanine-induced premature proboscis retraction. L-canavanine-induced premature proboscis retraction (PPR) was analyzed with 100 mM sucrose solution (white bars) and a solution containing 100 mM sucrose+40 mM L-canavanine (black bars). For all genotypes, the percentage of PPR is very low when tarsi are stimulated with the sucrose solution. Gr66a-Gal4/+, UAS-RNAiG α_{o47A} /+ (UAS-RNAiGo/+) and UAS-RNAiG α_{i65A} /+ (UAS-RNAiGi/+) control flies as well as Gr66a-Gal4/++;UAS-RNAiG α_{i65A} /+ (Gr66a-Gal4/++;UAS-RNAiGi/+) flies prematurely retract their proboscis when tarsi are in contact with a L-canavanine containing sucrose solution. On the contrary, the percentage of Gr66a-Gal4/++;UAS-RNAiG α_{o47A} /+ (Gr66a-Gal4/++;UAS-RNAiGo/+) flies that prematurely retracted their proboscis is very low, indicating that these flies maintained their proboscis extended due to L-canavanine detection defects. Error bars indicate SEM. Asterisks indicate significant differences by Unpaired Student's *t* test (ns: not significant, *** $p < 0.001$). doi:10.1371/journal.pone.0063484.g003

to mimics the GDP bound G α (G α^{GDP}) and which acts as a dominant negative of the G α^{GTP} form [28]. Note that the effect was stronger by using the G α_{o47A} RNAi than the G α^{GDP} construct (Fig. 4), likely because the RNAi was more efficient to block G α_{o47A} function. The same experiments were performed with flies expressing a RNAi construct against G α_{i65A} specifically in bitter-sensitive taste neurons. As shown in the Fig. S1.A, G α_{i65A} knock-down had no impact on L-canavanine detection, confirming the data obtained on PPR analysis. Altogether, these data indicate that L-canavanine detection requires the presence of G α_{o47A} , but not G α_{i65A} in bitter-sensitive taste neurons.

Pertussis toxin inhibition of G α_{o47A} strongly reduced L-canavanine aversion

To further demonstrate that G α_{o47A} is involved in L-canavanine detection, we took advantage of a transgenic line carrying the gene encoding for Pertussis toxin (PTX) under the control of UAS sequence. In vertebrates, PTX is known to specifically block the function of G α_i and G α_o proteins by catalyzing the ADP-ribosylation of these G proteins at a conserved C-terminal cysteine [39]. However, in *Drosophila melanogaster*, it is well established that PTX inhibits only G α_o , as the G α_i protein does not contain this cysteine [40]. We crossed the Gr66a-Gal4 line with the UAS-PTX line and analyzed the behavior of the progeny (Gr66a-Gal4/++;UAS-PTX/+) by using two-choice feed-

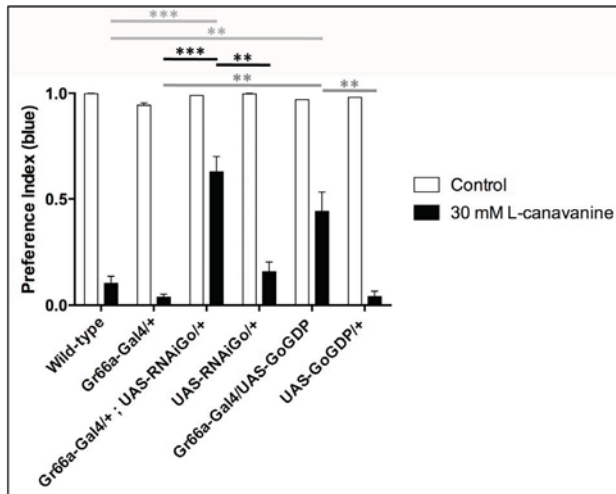


Figure 4. L-canavanine aversion is reduced when bitter-sensitive taste neurons express a RNAi construct against *G α o47A* or a dominant negative form of *G α o47A*. Two-choice feeding test experiments showing preference index (PI) for the blue solution of different genotypes. Control (white bars) and 30 mM L-canavanine (black bars) indicate that no drug or 30 mM L-canavanine was added to the blue solution, respectively. A complete preference or aversion is indicated by a PI value of 1 or 0, respectively. The down regulation of *G α o47A* by RNA interference (Gr66a-Gal4/+;UAS-RNAiGo/+) and the inhibition of *G α o47A* by using a dominant negative construct (Gr66a-Gal4/UAS-Go^{GDP}) reduced the aversion to L-canavanine compared to controls (wild-type, Gr66a-Gal4/+, UAS-RNAiGo/+ and UAS-Go^{GDP}/+). Note that all genotypes did not show any defect for sugar detection. Error bars indicate SEM. Asterisks indicate significant differences by Unpaired Student's *t* test (** *p*<0.01, *** *p*<0.001). doi:10.1371/journal.pone.0063484.g004

ing test experiments. As shown on Fig. 5A, the progeny of the parental lines crossed with wild-type flies had a normal taste behavior, *i.e.* Gr66a-Gal4/+ and UAS-PTX/+ flies detected and avoided to eat the L-canavanine containing sucrose solution (black bars). On the contrary, Gr66a-Gal4/+;UAS-PTX/+ flies did not detect at all the L-canavanine as they ate the L-canavanine containing blue solution at the same level that the blue solution that did not contain L-canavanine (compare black and white bars in Fig. 5A, respectively). One hypothesis that could explain this result is that blocking *G α o47A* function affects the development or the physiology of bitter-sensitive taste neurons. In order to exclude this hypothesis, we repeated the same experiment by using caffeine instead of L-canavanine. Caffeine is a potent repellent acting on bitter-sensitive taste neurons expressing GR66a [8]. As shown in Fig. 5A, Gr66a-Gal4/+;UAS-PTX/+ flies are strongly repelled by the presence of caffeine in the blue sucrose solution. This data strongly suggested that the impairment of *G α o47A* function did not alter the development or the differentiation of the bitter-sensitive taste neurons in Gr66a-Gal4/+;UAS-PTX/+flies.

In order to confirm these data, we performed electrophysiological studies on the s6 sensilla of the proboscis, which is known to respond to bitter compounds [7]. As shown in Fig. 5B and 5C, Gr66a-Gal4 and UAS-PTX parental lines responded to 40 mM L-canavanine and 10 mM caffeine at approximately the same level. In contrast, no response was observed during L-canavanine stimulation on Gr66a-Gal4/+;UAS-PTX/+ flies. These data were confirmed on Gr66a-Gal4/+;UAS-RNAiG α o47A/+ flies, which had a strongly reduced response to L-canavanine (Fig. 5B and 5C), revealing that *G α o47A* was required for L-canavanine perception. It is likely that the effect obtained by using PTX are stronger than

the ones obtained with the RNAi-G α o47A (or the *G α o^{GDP}* construct, see Fig. 4) because the PTX-induced blockade of *G α o* function is irreversible.

Importantly, we still detected a normal response during caffeine stimulation on Gr66a-Gal4/+;UAS-PTX/+ and Gr66a-Gal4/+;RNAiG α o47A/+ flies (Fig. 5B and 5C), confirming that their bitter-sensitive taste neurons were fully functional. To definitively exclude a role of *G α i65A* in L-canavanine detection, we performed spike recordings on Gr66a-Gal4/+;UAS-RNAi-G α i65A/+ flies and found no statistical significant differences compared to the Gr66a-Gal4 and UAS-RNAiG α i65A/+ control lines during L-canavanine or caffeine stimulation (Fig. S1B and S1C). Note that the decreased response observed between Gr66a-Gal4/+;UAS-RNAiG α i65A/+ and wild-type control flies during L-canavanine stimulation is very likely due to the UAS-RNAiG α i65A transgene insertion, which showed by itself a reduced response when crossed with wild-type control flies (Fig. S1B and S1C). Altogether, these behavioral and electrophysiological data show that PTX-induced *G α o47A* inhibition and RNAi knock-down of *G α o47A* strongly affect L-canavanine detection but have no effect on caffeine sensitivity.

Discussion

The goal of this study was to explore the L-canavanine-induced signaling transduction pathway in bitter-sensitive GRNs of *Drosophila*. By using a multidisciplinary approach, we provided evidence that *G α o47A* protein is required for L-canavanine detection.

Our study identified for the first time a *Drosophila* G protein subunit required for the detection of the toxic compound L-canavanine. Indeed, we demonstrated that rejection behavioral responses to L-canavanine (premature proboscis retraction and avoid eating) as well as electrophysiological response on proboscis sensilla known to respond to bitter compounds were dependent on active *G α o47A*. These results are important since they are supporting our previous report showing that DmXR, a *G α i_o* coupled mGluR-like GPCR, is mediating the repellent effect of L-canavanine. We have no explanation for the recent results of Lee and collaborators reporting that flies missing DmXR displayed normal L-canavanine avoidance [22]. To gain further insight on L-canavanine associated signal transduction, we explored the involvement of heterotrimeric G proteins, which are crucial downstream effectors of GPCR signaling. Here, the inactivation of *G α o47A* was obtained by different technical approaches, reducing a possible artifact. In addition, the behavioral and electrophysiological responses to caffeine were perfectly maintained in bitter-sensitive taste neurons in which *G α o47A* was either down-regulated by using a RNAi-G α o47A construct or blocked by using the pertussis toxin (PTX), excluding a general effect of the loss of *G α o47A* function on signaling events involved in bitter sensing in those neurons.

The GR family is likely not belonging to the GPCR family of receptors because recent studies have revealed that insect GRs, like their related ORs, have an inverted topology relative to GPCRs with their N-terminus being intracellular and their C-terminus extracellular [16]. GRs are likely channels. This idea is reinforced by the recent study of Sato and collaborators that found that BmGr-9, a GR from *Bombyx mori*, constitutes a ligand-gated ion channel responding to D-fructose [41]. In *Drosophila*, GR33a was described as a co-receptor for most bitter compounds [9] but we found no evidence that this receptor was involved in L-canavanine detection (data not shown). However, Lee and collaborators reported that GR66a and GR8a are required for

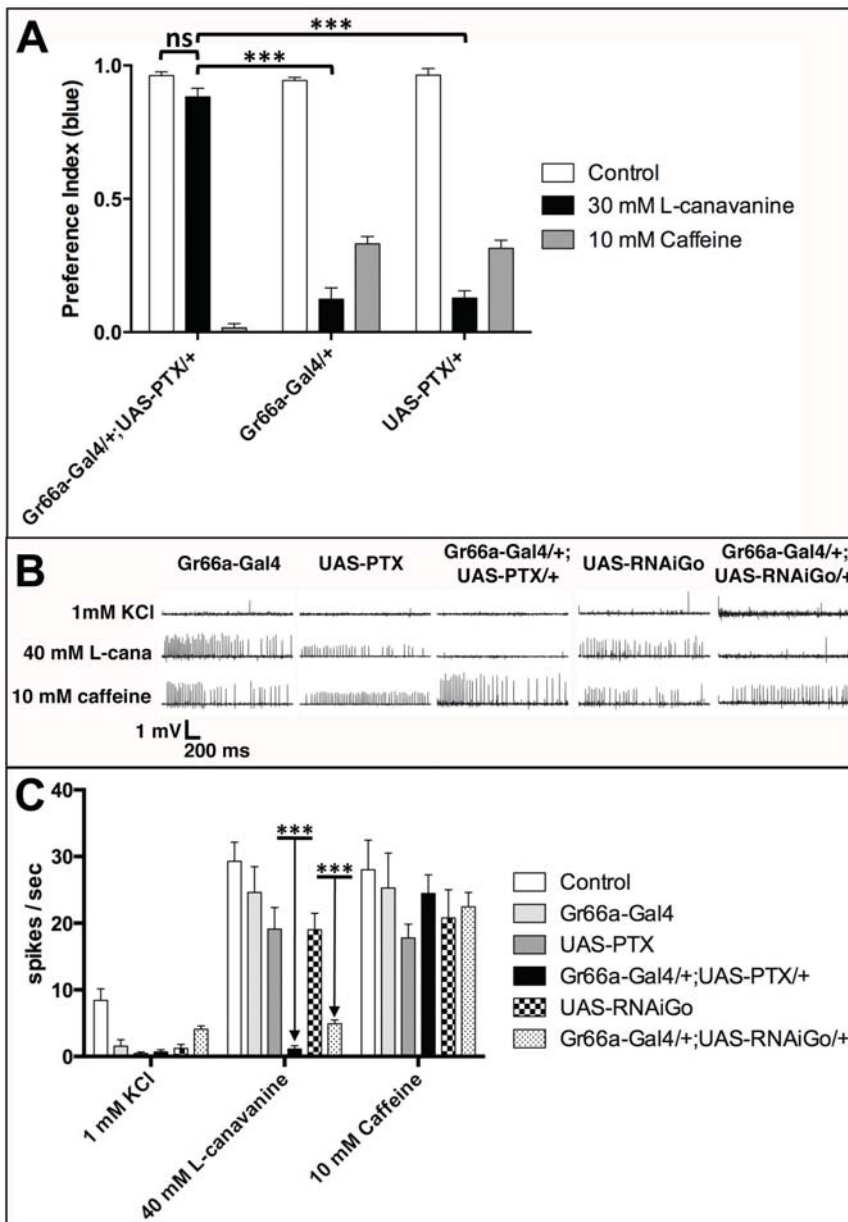


Figure 5. PTX inhibition of $G\alpha 47A$ in bitter-sensitive taste neurons highly reduces L-canavanine aversion and L-canavanine-induced nerve firings, but has no effect on caffeine aversion. A) Two-choice feeding test experiments showing preference index for the blue solution of flies with different genotypes. Control indicates that no drug was added to the blue medium (white bars). Data obtained by using 30 mM L-canavanine in the blue medium are shown in black bars. The expression of a selective toxin (pertussis toxin, PTX) for $G\alpha 47A$ in Gr66a-positive taste neurons (Gr66a-Gal4/+;UAS-PTX/+) highly reduces the aversion to L-canavanine compared to controls (Gr66a-Gal4/+ and UAS-PTX/+). Gr66a-Gal4/+;UAS-PTX/+ did not distinguish the control and the L-canavanine containing solutions (ns, $p=0.0526$). Note that Gr66a-Gal4/+;UAS-PTX/+ flies are more sensitive to caffeine (grey bar) than the Gr66a-Gal4/+ and UAS-PTX/+ control lines ($p<0.001$). Error bars indicate SEM. Asterisks indicate significant differences by Unpaired Student's t test (ns: not significant, *** $p<0.001$). **B–C)** Electrophysiological recordings were performed from $s6$ sensilla on the proboscis of flies with different genotypes. The electrical activity of the taste neurons was recorded by capping taste sensillum with an electrode containing 1 mM KCl as an electrolyte and the stimulus (40 mM L-canavanine or 10 mM caffeine). **B)** Sample responses for 1 mM KCl, 40 mM L-canavanine (mentioned as L-cana) and 10 mM caffeine on Gr66a-Gal4,UAS-PTX, Gr66a-Gal4/+;UAS-PTX/+, UAS-RNAiGo and Gr66a-Gal4/+;UAS-RNAiGo/+ flies. **C)** Compared to control (white bars) and parental lines (light grey, dark grey and squared bars), Gr66a-Gal4/+;UAS-PTX/+ (black bars) and Gr66a-Gal4/+;UAS-RNAiGo/+ (dotted bars) did not respond to 40 mM L-canavanine. Note that the response to 10 mM caffeine is not altered for all genotypes. The response was evaluated by counting the number of spikes elicited during the first second of the stimulation. $N=7-10$ for each condition. Error bars indicate SEM. Asterisks indicate significant differences by Unpaired Student's t test (***) $p<0.001$. doi:10.1371/journal.pone.0063484.g005

L-canavanine response [22]. Our experiments are not excluding that DmXR plus one or several GRs are required for a full response to L-canavanine. One hypothesis may be that L-canavanine binds to the GPCR DmXR that activates $G\alpha 47A$,

to finally stimulate a complex of GRs containing at least GR66a and GR8a. Another hypothesis could be that L-canavanine acts on GR8a/GR66a and that a DmXR-linked metabotropic mechanism influences the GR-mediated signal transduction. What is the

exact role of G α o and its downstream effectors remains to be determined. A second messenger can be involved but a direct binding of G α o47A and/or G β / γ subunits on GRs cannot be excluded [42]. A future challenge will be to identify the others players involved in L-canavanine detection.

Involvement of G proteins in bitter taste transduction was also found in other fly species. By using GDP β S, a competitive inhibitor of G-protein activation, Ouyang and collaborators found that strychnine and quinine detection in blowflies is dependent on a G protein signaling cascade [43]. While their approach did not allow them to unambiguously identify which subtype of G proteins is involved, their data suggest that the G protein-dependant signaling cascade is linked with the activation of phospholipase C and IP production, suggesting that the G protein involved there is a G α_q subtype. Several studies have found an involvement of *Drosophila* G protein subunits in the detection of sugars. These G proteins include G γ_1 [44], G α_s [45], G α_q [46] and also G α_o [47]. Interestingly, Bredendiek and collaborators found that G α_o function is required in sugar-sensitive GRNs for the detection of sucrose, glucose, and fructose, but not for trehalose and maltose [47]. Altogether, this suggests that different sugars may activate different signaling pathways within sugar-sensitive GRNs. So, it seems that, at least in sugar and bitter-sensitive GRNs, distinct ligand may activate distinct signaling pathways leading to neuronal activation. It is important to note that in all these studies, G proteins were not “essential” for the transduction mechanisms as the response for tastants were never fully abolished when G protein function was impaired. In our study, we showed that blocking G α_o function led to a very strong reduced response for L-canavanine, clearly indicating that G α_o is a crucial downstream effector for L-canavanine detection by *Drosophila* bitter-sensitive GRNs.

Within the large family of GPCRs, DmXR belongs to the class C, which includes the metabotropic glutamate receptors, the GABA_B receptors, the calcium-sensing receptor as well as some taste and pheromone receptors. The mX receptors form a distinct group within the mGluRs subclass [21]. In vertebrates, there are eight mGluRs that can be distinguished in three groups based on their sequence homology and pharmacology. While all mGluRs are well known for their roles in the central nervous system [48], recent studies suggest that mGluR1 and mGluR4 subtypes are involved in the umami response [49,50]. Umami taste, which is mostly elicited by L-glutamate, is also detected by heteromers of taste receptor type 1 (T1R1+T1R3) [51]. It is well known that the transduction cascade coupled to T1R1/T1R3 GPCRs relies on G proteins that will ultimately lead to the activation of the ion channel TRPM5 [52]. On the contrary, umami detection by mGluR1/4 seems to be independent of TRPM5 but the signaling cascade coupled to mGluR1/4 in taste buds remains to be elucidated [49]. It is interesting to note that these two mGluRs are coupled to different transduction pathways in heterologous systems: mGluR1 stimulates phospholipase C and phosphoinositide hydrolysis while mGluR4 inhibits adenylyl cyclase and cAMP production [33]. However, it could be that mGluR1 and mGluR4 form a heterodimer within taste buds and that this heterodimer has a unique coupling to G proteins. A future challenge will be to determine which G protein is required for umami detection in mice taste buds.

Most if not all bitter compounds previously used to study taste in insects, such as caffeine or quinine for example, lead to an inhibition of the proboscis extension reflex (PER) induced by sugar solution in contact with legs [38]. On the contrary, L-canavanine did not induce any inhibition of PER but rather a premature retraction of the proboscis (PPR), *i.e.* flies extend their proboscis

but retract it immediately. This rejection behavior is sufficient to avoid L-canavanine ingestion. This difference of behavior may be explained by the fact that L-canavanine acts on a GPCR while other bitter compounds act on ligand-gated GRs, the metabotropic pathway being slower than the ionotropic pathway. This point is difficult to answer yet as it was never shown that bitter compounds, such as caffeine or quinine, act directly on GRs. In conclusion, future exciting studies will help to decipher the complex signaling pathways involved in taste transduction in *Drosophila*.

Supporting Information

Figure S1 RNAi knockdown of Gai65A in bitter-sensitive taste neurons has no effect on L-canavanine and caffeine detection.

A) Two-choice feeding test experiments showing preference index for the blue solution of flies with different genotypes. Control indicated that no drug was added to the blue medium (white bars). Data obtained by using 30 mM L-canavanine or 10 mM caffeine in the blue medium are shown in grey and black bars, respectively. Compared to the Gr66a-Gal4/+ and UAS-RNAiGai65A/+ (UAS-RNAiGi/+) control lines, Gr66a-Gal4/+;UAS-RNAiGai65A/+ (Gr66a-Gal4/+;UAS-RNAiGi) flies did not show defect in L-canavanine aversion (ns $p = 0.0542$ and 0.6685 , respectively). Note that aversion to caffeine was comparable for the three genotypes. Error bars indicate SEM. Statistical significant differences were analyzed by Unpaired Student's *t* test (ns: not significant). **B–C)** Electrophysiological recordings were performed from s6 sensilla on the proboscis of flies with different genotypes. The electrical activity of the taste neurons was recorded by capping taste sensillum with an electrode containing 1 mM KCl as an electrolyte and the stimulus (40 mM L-canavanine or 10 mM caffeine). **B)** Sample responses for 1 mM KCl, 40 mM L-canavanine (mentioned as L-cana) and 10 mM caffeine on Gr66a-Gal4 parental line, UAS-RNAiGai65A/+ (UAS-RNAiGi/+) and Gr66a-Gal4/+;UAS-RNAiGai65A/+ (Gr66a-Gal4/+;UAS-RNAi/+) flies. **C)** No statistically significant differences were observed between Gr66a-Gal4/+;UAS-RNAiGai65A/+ (Gr66a-Gal4/+;UAS-RNAi/+, black bars) flies and the Gr66a-Gal4 parental line (light grey bars) as well as the UAS-RNAiGai65A/+ control flies (UAS-RNAiGi/+, dark grey bars) ($p = 0.154$ and 0.205 respectively). Note that a significant decrease of spike numbers is observed between UAS-RNAiGai65A/+ flies and Gr66a-Gal4 parental line as well as the control. This likely due transgene insertion effect explains why Gr66a-Gal4/+;UAS-RNAi/+ flies showed a significant decrease of spike numbers during L-canavanine stimulation compared to wild-type control flies (white bars). Note that the response to 10 mM caffeine is not statistically different between all genotypes. The response was evaluated by counting the number of spikes elicited during the first second of the stimulation. $N = 7–10$ for each condition. Error bars indicate SEM. Asterisks indicate significant differences by Unpaired Student's *t* test (ns: not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (TIF)

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Author Contributions

Conceived and designed the experiments: MLP FMP YG LS. Performed the experiments: ID MAA CM YG LS. Analyzed the data: ID MAA CM

MLP FMP YG LS. Contributed reagents/materials/analysis tools: ID
MAA CM MLP FMP YG LS. Wrote the paper: JB MLP FMP YG LS.

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B. Article 2 : Dual mechanism for bitter avoidance in *Drosophila*

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Abstract

In flies and humans, bitter chemicals are known to inhibit sugar detection, but the adaptive role of this inhibition is often overlooked. At best, this inhibition is described as contributing to the rejection of potentially toxic food, but no studies have addressed the relative importance of the direct pathway that involves activating bitter-sensitive cells versus the indirect pathway represented by the inhibition of sugar detection. Using toxins to selectively ablate or inactivate populations of bitter-sensitive cells, we assessed the behavioral responses of flies to sucrose mixed with strychnine (which activates bitter-sensitive cells and inhibits sugar detection) or with L-canavanine (which only activates bitter-sensitive cells). As expected, flies with ablated bitter-sensitive cells failed to detect L-canavanine mixed with sucrose in three different feeding assays (proboscis extension responses, capillary feeding, and two-choice assays). However, such flies were still able to avoid strychnine mixed with sucrose. By means of electrophysiological recordings, we established that bitter molecules differ in their potency to inhibit sucrose detection and that sugar-sensing inhibition affects taste cells on the proboscis and the legs. The optogenetic response of sugar-sensitive cells was not reduced by strychnine, thus suggesting that this inhibition is linked directly to sugar transduction. We postulate that sugar-sensing inhibition represents a mechanism in insects to prevent ingesting harmful substances occurring within mixtures.

Dual Mechanism for Bitter Avoidance in *Drosophila*

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In flies and humans, bitter chemicals are known to inhibit sugar detection, but the adaptive role of this inhibition is often overlooked. At best, this inhibition is described as contributing to the rejection of potentially toxic food, but no studies have addressed the relative importance of the direct pathway that involves activating bitter-sensitive cells versus the indirect pathway represented by the inhibition of sugar detection. Using toxins to selectively ablate or inactivate populations of bitter-sensitive cells, we assessed the behavioral responses of flies to sucrose mixed with strychnine (which activates bitter-sensitive cells and inhibits sugar detection) or with L-canavanine (which only activates bitter-sensitive cells). As expected, flies with ablated bitter-sensitive cells failed to detect L-canavanine mixed with sucrose in three different feeding assays (proboscis extension responses, capillary feeding, and two-choice assays). However, such flies were still able to avoid strychnine mixed with sucrose. By means of electrophysiological recordings, we established that bitter molecules differ in their potency to inhibit sucrose detection and that sugar-sensing inhibition affects taste cells on the proboscis and the legs. The optogenetic response of sugar-sensitive cells was not reduced by strychnine, thus suggesting that this inhibition is linked directly to sugar transduction. We postulate that sugar-sensing inhibition represents a mechanism in insects to prevent ingesting harmful substances occurring within mixtures.

Key words: behavior; bitter; *Drosophila*; electrophysiology; mixture interaction; sweet

Introduction

Animals, including insects, detect a number of chemicals as aversive via specialized taste cells (Yarmolinsky et al., 2009). These aversive chemicals define a sensory space usually described as “bitter” by analogy with human sensation. Bitter taste is thought to contribute to protecting animals against ingesting potentially toxic molecules (Glendinning, 2002, 2007). Accordingly, omnivorous/polyphagous animals possess a large panel of bitter taste receptors, whereas animals specialized on a restricted diet have few, because they are potentially exposed to a lesser variety of toxic molecules. This rule has been observed in vertebrates (Wooding, 2005; Dong et al., 2009; Jiang et al., 2012; Hong and Zhao, 2014; Li and Zhang, 2014) and insects (McBride and Arguello, 2007; Richards et al., 2008; Kirkness et al., 2010), with a few exceptions (Wanner and Robertson, 2008; Engsontia et al., 2014). Animals, including insects, also possess “sweet” receptors expressed in specific taste cells that detect molecules triggering food acceptance (Zhao et al., 2003; Chandrashekar et al., 2006; Yarmolinsky et al., 2009). These two populations of taste cells that sense either sugar or bitter chemicals directly trigger feeding or aversion if activated (Marella et al., 2006; Hiroi et al., 2008; Gordon and Scott, 2009), whereas disrupting one of them impairs the corresponding behavior (Wang et al., 2004). This suggests that taste is encoded by labeled lines and supports the view that the final decision is taken after the brain has weighed information from these two lines (Masek and Scott, 2010).

However, each taste modality is not insensitive to stimuli from other modalities. For example, bitter chemicals inhibit sugar detection in insects of different groups (Morita, 1959; Dethier, 1980, 1987; Schoonhoven, 1982; Dethier and Bowdan, 1989, 1992; Chapman et al., 1991; Schoonhoven and Liner, 1994), including *Drosophila* (Meunier et al., 2003a; Lee et al.,

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*A.S.F. and M.-J.S. contributed equally to the work.

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Table 1.

Fly strain	Short-hand name	Significance to the study	Source
<i>White</i>	<i>w¹¹¹⁸</i>	Reference strain carrying the mutation <i>white</i>	K. Scott
	<i>Gr66a–Gal4</i>	Driver for cells expressing <i>Gr66a</i> (bitter-sensitive)	K. Scott (Wang et al., 2004)
	<i>Gr64f–Gal4</i>	Driver for cells expressing <i>Gr64f</i> (sugar-sensitive)	J. Carlson (Dahanukar et al., 2007)
	<i>Gr33a–Gal4</i>	Driver for cells expressing <i>Gr33a</i> (bitter-sensitive)	J. Carlson
	<i>UAS–DTI</i>	Reporter expressing DTI to kill cells	K. Scott (Wang et al., 2004)
<i>w*</i> ; <i>P[UAS–TeTxLC.tnt]R3</i>	<i>UAS–TeTxLC</i>	Reporter expressing TeTxLC to inactivates synaptic transmission	Bloomington <i>Drosophila</i> Stock Center strain BL 28997
<i>w*</i> ; <i>P[UAS–H134R–Chr2]2</i>	<i>UAS–Chr2</i>	Reporter expressing a channel rhodopsin to activate neurons with light	Bloomington <i>Drosophila</i> Stock Center strain BL 28995
<i>Gr66a–Gal4/UAS–Chr2</i>	<i>Gr66a>Chr2</i>	Express Chr2 into <i>Gr66a</i> cells	
<i>Gr64f–Gal4/UAS–Chr2</i>	<i>Gr64f>Chr2</i>	Express Chr2 into <i>Gr64f</i> cells	
<i>Gr66a–Gal4/+;UAS–DTI/+</i>	<i>Gr66a>DTI</i>	Express DTI into <i>Gr66a</i> cells to kill them	
<i>Gr66a–Gal4/+;UAS–TeTxLC/+</i>	<i>Gr66a>TeTxLC</i>	Express the TeTxLC into <i>Gr66a</i> cells to inactivate their synaptic transmission	

2010, 2012; Sellier and Marion-Poll, 2011; Jeong et al., 2013) in which other interactions were described recently, such as between sugars and acids (Charlu et al., 2013; Chen and Amrein, 2014). Whereas the molecular basis of bitter suppression of sugar detection is still under scrutiny and may involve odorant binding proteins (OBPs; Jeong et al., 2013; Swarup et al., 2014), the behavioral role of such interactions have been considered rarely (König et al., 2014).

We examined here to what extent bitter suppression of sugar detection influences behavioral choices in adult *Drosophila*. We demonstrate that the activation of bitter-sensitive cells does not suffice to inhibit proboscis extension responses (PERs) and that complete inhibition of the PER requires shutting off the signal conveyed by sugar-sensitive cells. Using behavior and optogenetics, we show that the simultaneous activation of bitter-sensitive cells along with the inhibition of sugar detection is necessary to elaborate a proper response to ambiguous mixtures of tastants. We further show that sugar-sensitive cell inhibition is a specific property of sugar transduction. We postulate that sugar-sensing inhibition complements canonical bitter detection to prevent the ingestion of harmful substances when mixed with beneficial substances.

Materials and Methods

Chemicals. Sucrose, KCl, L-canavanine, caffeine, lobeline, escin, nicotine, strychnine, denatonium, tricholine citrate (TCC), erioglaucine, sulforhodamine B, and all *trans*-retinal were from Sigma-Aldrich. Brilliant blue FCF was from Tokyo Kasei.

Flies. Flies were maintained on a standard cornmeal agar medium at 25°C and 80% humidity on a 12 h light/dark cycle. Unless notified otherwise, we used 2- to 5-d-old adults. Insects subjected to behavioral tests were starved for 24 h before the experiment.

To manipulate specific populations of taste cells, we used the UAS–Gal4 system (Brand and Perrimon, 1993). Bitter-sensitive cells were targeted using *Gr66a–Gal4* (Wang et al., 2004). Sugar-sensitive cells were addressed with *Gr64f–Gal4* (Dahanukar et al., 2007). We crossed these flies with flies carrying a secondary transgene activated by GAL4 in the progeny. As a secondary transgene, we used a diphtheria toxin (*UAS–DTI*; Wang et al., 2004) to ablate cells expressing *Gr66a* in the progeny (called *Gr66a>DTI* throughout text). We also used the tetanus toxin light chain (*TeTxLC*), which impairs neurotransmission at synapses by degrading synaptobrevin (Sweeney et al., 1995). In the progeny of *Gr66a–Gal4* flies crossed with *UAS–TeTxLC* flies and *Gr66a–Gal4/+;UAS–TeTxLC/+* flies (mentioned as *Gr66a>TeTxLC* throughout text), bitter-sensitive neurons are inactive. To express a channel rhodopsin into taste neurons, *UAS–Chr2* flies were crossed with flies carrying *Gr66a–Gal4* (Wang et al., 2004) or *Gr64f–Gal4* (Dahanukar et al., 2007). In the progeny (mentioned as *Gr66a>Chr2* and *Gr64f>Chr2* throughout text), blue light (BL) induces a spiking activity in the neurons expressing channelrhodopsin 2 (*Chr2*). These flies and the control strains

were raised on medium supplemented with 1 mM all *trans*-retinal (Schroll et al., 2006; Hornstein et al., 2009) and kept in darkness.

w¹¹¹⁸, *Gr66a–Gal4*, and *UAS–DTI* strains were generously provided by Kristin Scott (University of California at Berkeley, CA) and *Gr64f–Gal4* flies by John Carlson (Yale University, New Haven, CT). *UAS–TeTxLC* and *UAS–Chr2* flies were obtained from the Bloomington *Drosophila* Stock Center (strains BL 28997 and BL 28995, respectively). A list of the flies used in this study is provided in Table 1. In all experiments, we compared flies carrying both transgenes (*UAS* and *GAL4*) to their parents carrying only one transgene and designed hereafter as control flies.

Proboscis responses (PER and proboscis retraction) after stimulation of labellar sensilla. To stimulate labellar taste sensilla, flies (1–5 d old) were prepared according to Shiraiwa and Carlson (2007). Before the experiment, flies were starved for 24 h by placing them into a vial with humid cotton. Flies were introduced into the cut ends of 200 μ l micropipette tips so that the head was protruding and the proboscis was free to move. Taste sensilla of the labellum were stimulated by gently touching the labellum during 2 s with the fine tapering end of a strip of filter paper, soaked with the test solution (Fig. 1A); if the fly extended its proboscis, the stimulus was removed immediately to prevent drinking. We scaled the PER of the fly as 1 if the proboscis was fully extended within 2 s after the contact and as 0 otherwise. We also monitored proboscis retraction (PR) and scaled it as 1 if the fly retracted the proboscis (and maintained retraction) within 5 s after having fully extended it and 0 otherwise (Fig. 1A; Dethier, 1980; Mitri et al., 2009).

We used this method to test whether ablating cells expressing *Gr66a* affects the responses to sugar mixed with strychnine or L-canavanine, which are both aversive for flies (Meunier et al., 2003a; Hiroi et al., 2004; Mitri et al., 2009; Sellier et al., 2011; Lee et al., 2012; Devambez et al., 2013). Strychnine is an alkaloid molecule, whereas L-canavanine is a toxic nonprotein amino acid (Rosenthal, 2001; Kool, 2005). Both substances activate bitter-sensitive cells in *Drosophila* and do not activate other taste cells (Meunier et al., 2003a; Hiroi et al., 2004; Lee et al., 2012). Therefore, it is expected that flies deprived of their bitter-sensitive cells should become less sensitive to these substances. Furthermore, there should be a discrepancy between the effect of bitter-sensitive cell ablation on L-canavanine and strychnine avoidance given that the former does not strongly inhibit sugar-sensitive cells (Jeong et al., 2013).

The sequence of stimulation was water, sucrose, test solution (strychnine plus sucrose, L-canavanine plus sucrose, or sucrose alone), sucrose, and water, each separated by 2 min. The responses to the test stimulus were recorded from only those flies that responded to the first sucrose presentation and that did not respond to water. The concentrations of chemicals used were 0.1 M sucrose, 10 mM strychnine plus 0.1 M sucrose, and 40 mM L-canavanine plus 0.1 M sucrose.

Proboscis responses (PER and PR) after stimulation of leg sensilla. To stimulate taste sensilla of the prothoracic legs, four to five flies (narcotized with ice) were disposed on a microscopic slide, placed on pads of adhesive clay (UHU yellow patafix), and restrained on their dorsum with fine strips of tape. They were left to recover from the manipulation at 25°C and 80% humidity for 2 h. Before the experiment, flies were fed water to satiation, and their legs were washed with water. The legs were

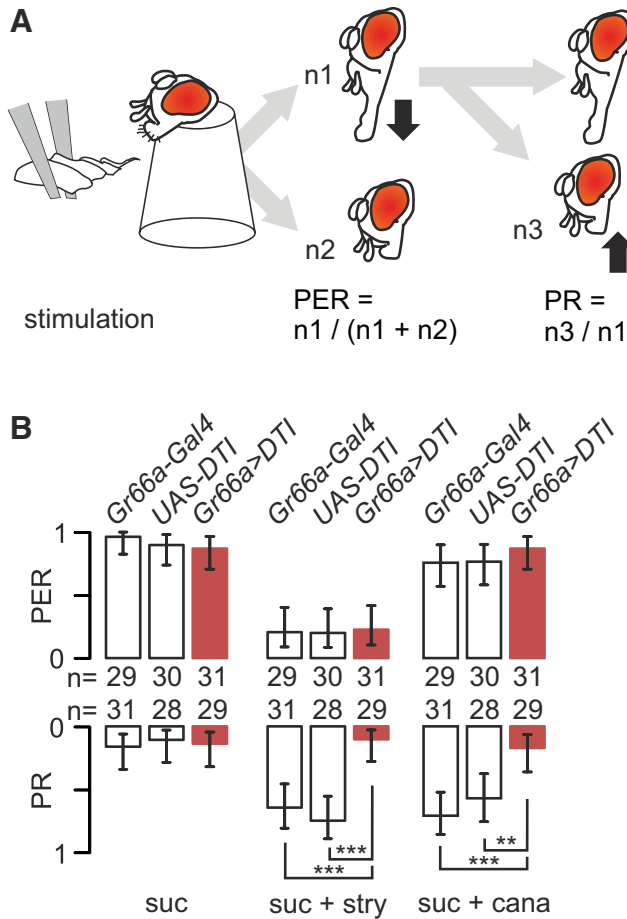


Figure 1. Strychnine-induced inhibition of the PER is not affected by the ablation of labellar taste neurons expressing *Gr66a*, whereas PR activation is reduced. **A**, The proboscis of restrained flies was stimulated with a wick soaked with test solution for 5 s. We monitored the number of flies extending their proboscis and the number of flies actively retracting their proboscis after having extended it within the 5 s observation period. **B**, Bitter-sensitive cells were ablated in the progeny of flies carrying a diphtheria toxin (*UAS-DTI*) crossed with flies carrying *Gr66a-Gal4*. We compare the PER (top bars graph) and the PR (bottom bars graph) of flies with ablated bitter cells (*Gr66a>DTI*, red bars) to control flies bearing only one of these constructions (*Gr66a-Gal4* or *UAS-DTI*, white bars). Each fly was stimulated in sequence with 0.1 M sucrose (suc), 0.1 M sucrose plus 10 mM strychnine (suc + stry) or 0.1 M sucrose + 40 mM L-canavanine (suc + cana). The values are displayed as the frequency of flies that responded \pm 95% binomial confidence intervals, and asterisks indicate the significance values from a Fisher's exact test when comparing strains ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$). The respective number of flies tested is displayed as numbers between the two graphs.

stimulated by touching them gently for 5 s with a toothpick dipped previously into a test solution. If the solution applied was a mixture, only one leg was touched (Fig. 2A). If bitter and sugar solutions were applied separately, one leg was stimulated with sugar and the contralateral leg was stimulated with bitter (or with water; Fig. 2A). Flies that did not extend their proboscis in response to the first presentation of sucrose were discarded. PERs and PRs were noted as mentioned previously.

This method was used to evaluate whether the mode of presentation of the stimuli had an influence on PER/PR and whether ablating different populations of bitter-sensitive cells of *Gr66a>DTI* or *Gr33a>DTI* flies would affect the responses. The order of stimulation was water, sucrose, test, water, and sucrose, separated by 2 min, at the same concentrations and with the same chemicals as in the previous experiment mentioned above.

PERs/PRs to concurrent optogenetic and chemical stimulations. We used a *Chlamydomonas reinhardtii* cation channel *ChR2* (Nagel et al., 2003) to activate taste cells expressing the protein encoded by this gene. *ChR2* channels are activated by light at a wave length of 488 nm (Schroll et al.,

2006). BL was delivered by a light fiber from a 480 nm light source LED (CoolLED pE-100; Scientifica) illuminating the whole animal. Flies expressing *ChR2* into either bitter-sensitive cells (using *Gr66a-Gal4*) or sugar-sensitive cells (using *Gr64f-Gal4*) were tested to evaluate whether the optogenetic activation of one class of taste cells could be modulated by the chemical activation of the other class.

To evaluate the effect of activating bitter-sensitive cells (expressing *Gr66a*) on PERs/PRs to sucrose, we stimulated *Gr66a>ChR2* flies and their parental lines with 0.1 M sucrose alone or concurrently with a spot of BL during 5 s (Fig. 3A). The order of stimulation was sucrose, water, test (sucrose or sucrose plus BL), water, and sucrose. To evaluate the effect of strychnine on the PERs/PRs to the optogenetic activation of sugar-sensitive cells, we stimulated *Gr64f>ChR2* flies with a spot of BL during 5 s and presented 10 mM strychnine concurrently (Fig. 3B). The order of stimulation was BL, BL plus water (control for tactile stimulation), test (BL or BL plus strychnine), water, and BL plus water. *Gr64f>ChR2* flies that did not respond to the first BL stimulation and to BL plus water were discarded. The control strains were unresponsive to BL.

Multiple choice capillary feeder test. Multiple choice capillary feeder (MultiCAFE) experiments were performed as in the study by Sellier et al. (2011). Female flies (1–2 d old) were fed fresh food for 1 d and then starved for 20–22 h in the presence of a disk of water-saturated filter paper. Groups of 20 flies were then introduced into a plastic box and given access to an array of capillaries (5 μ l, 32 mm; Hirschmann Geräte) containing food solutions for 2 h (in the dark; 25°C, 70% humidity). The liquid level in each capillary was measured using NIH ImageJ (Rasband and Bright, 1995) from pictures taken just before and at the end of the experiment. To assess evaporation, an additional box containing no flies was prepared. This box was prepared and handled in a similar manner, and the average change in liquid level, corresponding to evaporation, was subtracted from consumption measurements obtained from boxes containing flies. This test was used to assess whether *Gr66a>DTI* and *Gr66a>TeTxLC* flies are defective in the detection of different concentrations of strychnine and L-canavanine mixed with sucrose.

Two-choice feeding test. Flies (3–5 d old) were starved on water-saturated cotton for 24 h and then placed in groups of 100 on a 60-well microtiter plate (Dutscher) at 25°C for 2 h in the dark. Alternating wells contained either 1% agarose with 0.15 mg/ml erioglaucine dye (blue) and 5 mM sucrose (and 10 mM strychnine or 40 mM L-canavanine) or 0.2 mg/ml sulforhodamine B dye (red) and 1 mM sucrose. After 2 h, the flies were frozen and sorted according to abdomen color blue (B), red (R), or purple (P). A preference index (PI) for the blue solution was computed as $(NB + 0.5NP)/(NB + NP + NR)$ where N stands for the number of flies of each category. A PI value of 1 indicates complete preference for the 5 mM sucrose (blue) solution, whereas 0 indicates complete preference for the 1 mM sucrose (red) solution. To check that the food colors are not introducing a bias, the same experiment was performed after exchanging the colors. This test was used to evaluate whether *Gr66a>TeTxLC* flies are defective in the detection of sugar mixed with either 10 mM strychnine or 30 mM L-canavanine.

Electrophysiological recordings from taste sensilla on the proboscis. Taste sensilla were recorded from the proboscis of 1- to 5-d-old female flies. Flies were immobilized with fine strips of tape on a pad of clay (UHU yellow patafix) to maintain the proboscis extended and to expose sensilla of the labellum. The fly's body was electrically connected to the ground through either a glass electrode containing 0.1 mM KCl or a drop of electrocardiogram gel connected to a silver wire. Individual taste sensilla (I5, s6, and i9; see Fig. 5B–E) were stimulated for 2 s with a capillary electrode containing the stimulus and an electrolyte. This electrolyte was either 1 mM KCl or 30 mM TCC to inhibit the activity from the water cell (Wieczorek and Wolff, 1989). The recording electrode was connected to a taste-specific amplifier (Marion-Poll and van der Pers, 1996), further amplified 50–100 times, bandpass filtered at 10–2800 Hz (CyberAmp 320; Molecular Devices), and digitally sampled at 10 kHz (DT9816; Data Translation) under the control of a custom program, dbWave. The intensity of the response was measured by counting the number of spikes occurring during the 1 s of each recording. Stimulations were separated by at least 1 min. Spikes were not sorted by amplitude or shape, except noted otherwise, because extracellular recordings from taste receptors in

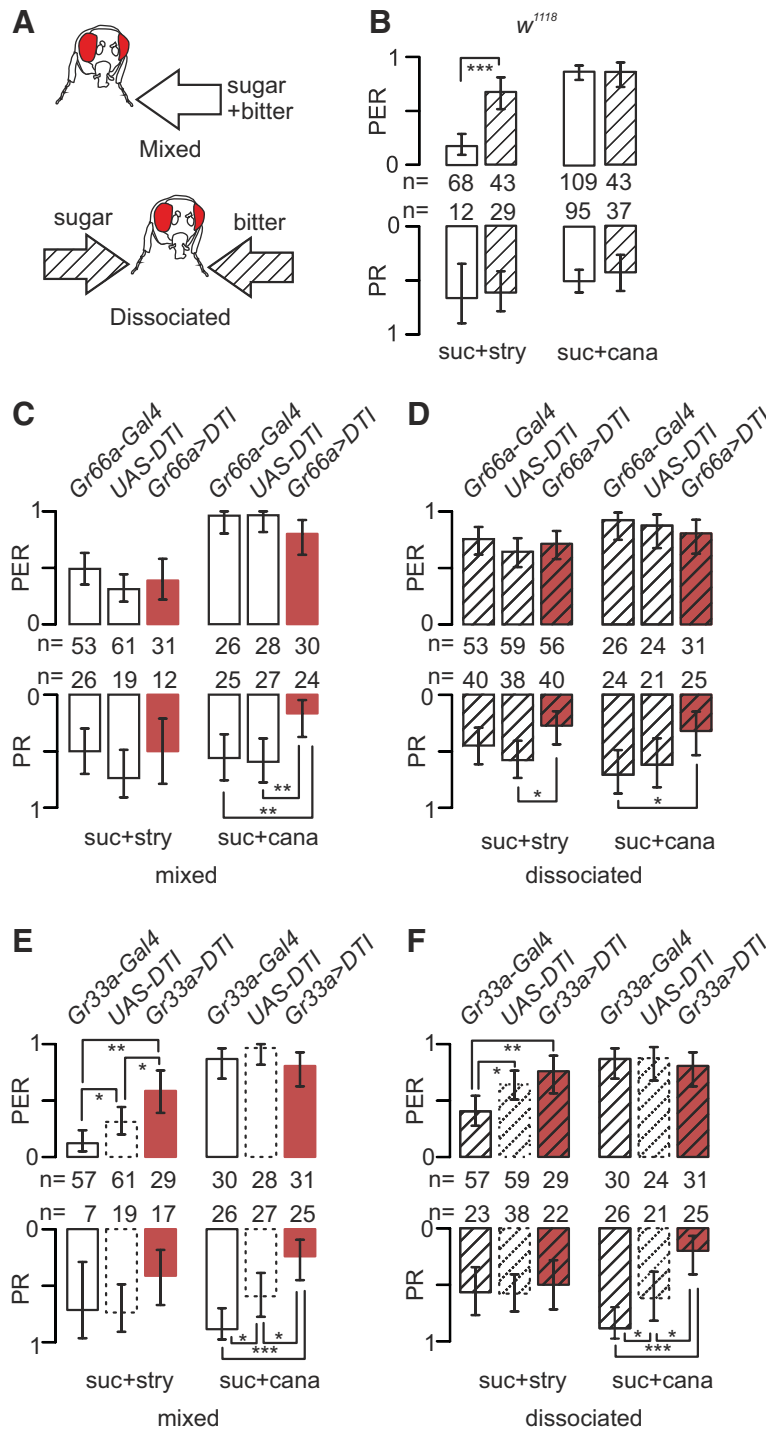


Figure 2. Strychnine-induced inhibition of the PER on the legs is more potent in a mixture with sugar than when presented separately, and bitter-cell ablation reduces the PR. **A**, Flies were stimulated with 100 mM sucrose and a bitter chemical during 5 s by gently touching one leg with a mixture (“mixed” presentation) or each tarsus separately with sugar on one side and bitter on the other side (“dissociated” presentation). The bitter stimulus was 100 mM sucrose plus 10 mM strychnine (suc + stry) or 100 mM sucrose plus 40 mM L-canavanine (suc + cana). **B**, *w¹¹¹⁸* flies respond more strongly in the PER to the mixed (white bars) than to the dissociated stimuli (striped bars) for suc + stry ($p < 0.001$) but not to sucrose plus L-canavanine. PRs are not different with both stimuli. **C, D**, We ablated cells expressing Gr66a and recorded the responses to the same stimuli in *Gr66a>DTI* flies (red bars) and in the parental strains that have intact taste cells (*Gr66a-Gal4* and *UAS-DTI*). **C**, PERs/PRs to mixed presentation. **D**, PERs/PRs to dissociated presentation (striped bars) for suc + stry ($p < 0.001$) but not to sucrose plus L-canavanine. PRs are not different with both stimuli. **E, F**, We also ablated cells expressing Gr33a and recorded the responses to these stimuli in *Gr33a>DTI* flies (red bars) and *Gr33a-Gal4* flies (with intact taste cells). *UAS-DTI* flies were not tested again, but the previous values are reported on the graph for convenience. **E**, PERs/PRs to the mixed presentation. **F**, PERs/PRs to the dissociated stimuli. Graph legends, statistical tests, and abbreviations are the same as in Figure 1.

Drosophila are notoriously difficult to sort (Fujishiro et al., 1984; Meunier et al., 2003b).

Three separate experiments were performed. In the first (see Fig. 5F–H), we looked at the relative effectiveness of seven bitter chemicals on the responses of i9, l5, and s6 sensilla to 0.1 M sucrose mixed or not with 1 mM of a bitter chemical (nicotine, caffeine, escin, lobeline, strychnine, and denatonium) or 40 mM L-canavanine. In a second experiment (see Fig. 5I–K), we looked at the effect of strychnine concentration (0.001, 0.1, and 10 mM) to inhibit the responses to 0.1 M sucrose in these sensilla.

In a third experiment (see Fig. 6), we recorded the spiking activity from l5, i9, and s6 sensilla in response to sucrose (0.01, 0.1, and 1 M), strychnine (0.001, 0.1, and 10 mM), and 0.1 M sucrose mixed with strychnine (0.001, 0.1, and 10 mM) in *Gr66a>DTI* flies and in their parental lines. Because i9 sensilla are not very sensitive to strychnine (Weiss et al., 2011), we also tested caffeine (0.001, 0.1, and 10 mM) to validate that the genetic construction is ablating the cell responding to bitter substances with the *Gr66a-Gal4* driver.

Electrophysiological recordings from taste sensilla on the legs. Taste sensilla from the legs were recorded using the same procedures as when recording from labellar sensilla, except that one front leg was immobilized and properly oriented to allow tip recording. To verify that expression of DTI in Gr66a-expressing cells abolished responses to bitter tastants, the sensilla f5b and f5s (Ling et al., 2014), which differ in their expression of Gr66a (see Fig. 7B), were stimulated with 10 mM strychnine and 40 mM L-canavanine.

Electrophysiological recordings and optogenetic stimulation. To combine chemical and optogenetic activation of taste neurons, we used a modified tip-recording configuration. To control more strictly the timing of the chemical stimulation, we placed the head stage of the amplifier and the stimulus electrode on a sliding platform driven by a piezoelectric linear actuator (PPA10M and amplifier CA μ 10; Cedrat Technologies). A light stimulator (480 nm blue light; model pE-100; CoolLED) and the piezo actuator were driven by a programmable logic controller (RIO-47122; Galil).

To test whether the firing activity of bitter-sensitive cells could affect the responses to sugar, we recorded from i9 sensilla in *Gr66a>ChR2* flies; these sensilla host only two taste cells that elicit extracellular action potentials that are easy to sort (Hiroi et al., 2004). Each recording lasted 6 s, during which three flashes of BL were delivered (1, 0.5, and 0.5 s separated by 0.5 s), starting 1 s after the beginning of the contact (see Fig. 8A). We displayed the time course of the responses within 100 ms bins.

To test whether strychnine inhibits the spiking generator within the sugar-sensitive cells or whether it interferes directly with sugar transduction, we recorded the responses to sugar or to optogenetic stimulation in the presence of strychnine in i9 and in l5/l7 sensilla of *Gr64f>ChR2* flies. The recording configura-

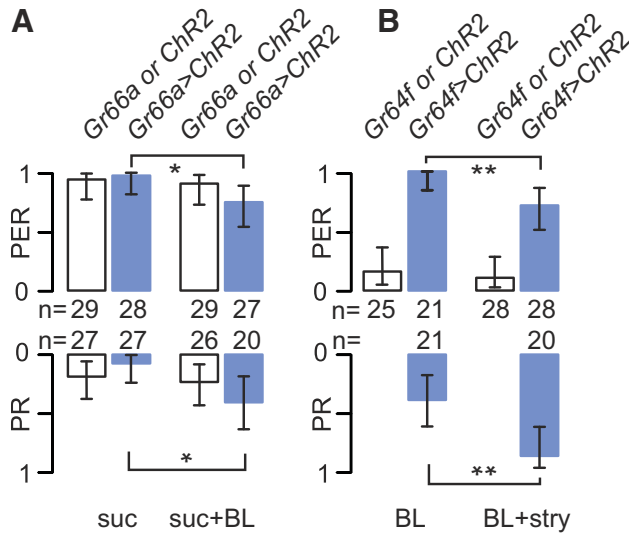


Figure 3. Optogenetic activation of either cell type bypasses the mixture effect. **A**, Flies expressing ChR2 in bitter-sensitive cells (*Gr66a>ChR2*) were stimulated on the tarsi with 0.1 M sucrose (suc) and then with 0.1 M sucrose plus BL (suc + BL) to activate bitter-sensitive cells. Data from *Gr66a>ChR2* flies are displayed in blue, and those from control strains (*Gr66a-Gal4* and *UAS-ChR2*; noted as *Gr66a* and *ChR2*, respectively) are in white. We observed a reduction of PERs in *Gr66a>ChR2* flies in response to suc + BL ($p = 0.02$, Fisher's exact test) but not in the control strains ($p = 1.00$). We also observed a significant increase in the PR in *Gr66a>ChR2* flies in response to suc + BL ($p = 0.01$) but no increase in the control strains ($p = 0.745$). **B**, Flies expressing ChR2 in sugar-sensitive cells (*Gr64f>ChR2*) were stimulated with BL alone to activate the sugar-sensitive cells (BL) and then with BL plus 10 mM strychnine to activate sugar- and bitter-sensitive cells (BL + stry). We observed a reduction of the PER in *Gr64f>ChR2* flies in response to BL ($p = 0.007$) but not in the control strains ($p = 0.695$) that were not reacting to BL. We also observed a strong increase of the PR in response to BL in *Gr64f>ChR2* flies ($p = 0.0036$). Graph legends, statistical tests, and abbreviations are the same as in Figure 1.

tion was the same as before, but the recording (and chemical stimulation) was limited to 2 s. The stimuli used were strychnine (1, 5, and 10 mM) "mixed" with BL or 0.1 M sucrose.

Statistical analysis. The proboscis responses (PERs and PRs) were compared with a Fisher's exact test (two tailed) using R 3.0.2 (R Foundation for Statistical Computing) or Statistica 10 (StatSoft). A 95% binomial confidence interval was calculated using JavaStat (<http://statpages.org/confint.html>).

We analyzed the statistical significance of MultiCAFE consumption as in the study by Sellier et al. (2011), using generalized linear mixed models (GLMMs), which provide a flexible approach to correct potential problems of correlation and heteroscedasticity. To model consumption in the MultiCAFE experiment, we used a Gaussian distribution under SAS 9.2 (SAS Institute).

Two-choice feeding tests were analyzed with unpaired Student's *t* tests with unequal variances to check for significant differences between pairs of data using Statistica 10.

For electrophysiology, we analyzed the data differently according to the questions asked. To compare the firing rate of sensilla stimulated by sugar and sugar plus bitter (see Fig. 5F–H), we used Wilcoxon's matched pairs tests. For other binary comparisons (see Figs. 5I–K, 7B, C, 8C, E), we used Kruskal–Wallis tests. Last, we used a one-way ANOVA with Fisher's least significant difference (LSD) *post hoc* analysis to evaluate the effect of strychnine on optogenetic responses (see Fig. 9A, B). These tests were performed using Statistica 10.

To analyze dose–response curves of our electrophysiological experiments (see Figs. 6, 7D, E), we fitted GLMMs using SAS 9.2 with a Poisson distribution with overdispersion to model the discrete nature of the number of spikes per second. For sucrose, strychnine, and the mixture of sucrose and strychnine, the models also consider a variance–covariance matrix with an autoregressive structure to account for correlations, because each insect was stimulated with the different concentrations. We

partitioned interactions constructing slice *F* tests and evaluated pairwise differences within single effects with *t* tests. All these outputs were generated using the GLIMMIX procedure of SAS 9.2.

Results

PERs/PRs to stimulation of the labellum with mixtures of sugar/bitter molecules

Because L-canavanine and strychnine are considered as bitter for *Drosophila*, we expected that both substances would reduce PERs. When stimulated with sucrose, flies responded to sucrose with a strong PER (Fig. 1B). In response to 10 mM strychnine mixed with 0.1 M sucrose, *Gr66a>DTI* flies and their parents showed a strongly reduced PER compared with 0.1 M sucrose alone ($p < 0.001$, Fisher's exact test), whereas L-canavanine had no effect. Only 10–15% of control flies exhibited a PR in response to 0.1 M sucrose (Fig. 1B). Approximately 60–70% of the flies from control strains retracted their proboscis in response to sucrose plus strychnine or L-canavanine ($p < 0.001$). However, *Gr66a>DTI* flies responded to the mixture at a level similar to sucrose alone ($p = 1$).

Altogether, these data indicate that strychnine differs from L-canavanine when mixed with sugar, with strychnine inhibiting PER but not L-canavanine, whereas both substances induce the PR. Although PERs seem to be insensitive to the presence or absence of *Gr66a* cells, the PR is affected strongly by the ablation.

PERs/PRs to stimulation of the legs with mixed or dissociated solutions of sugar/bitter molecules

We then examined whether similar findings could be obtained by stimulating leg taste receptors. Stimulating these appendages provides us with an additional possibility, which is to present sugar and bitter separately (Fig. 2A). This was tested on *w¹¹¹⁸* flies, on which we compared PERs/PRs to sugar and strychnine or L-canavanine by stimulating either one leg with the mixture (mixed presentation) or one leg with a bitter substance and the contralateral one with sugar (dissociated presentation; Fig. 2B). In these conditions, we obtained a highly significant difference of PER between strychnine plus sucrose versus strychnine plus L-canavanine in the mixed presentation ($p < 0.000$, Fisher's exact test, two tailed) but not in the dissociated presentation ($p = 0.07$). This suggests that the mixture of sugar and strychnine is more effective in preventing the initiation of a PER than when the stimuli (at the same concentration) are presented on different legs, even if we can expect to stimulate the same number of taste receptor neurons in both situations.

We then performed the same experiment in *Gr66a>DTI* flies and their parents (Fig. 2C, D). As expected, the strychnine-induced inhibition of the PER was more marked in the mixed mode than in the dissociated presentation mode in the control strains (Fig. 2C, D; $p < 0.001$) and *Gr66a>DTI* flies ($p = 0.006$). With L-canavanine, the situation was different as the PERs were similar in the two modes of presentation (Fig. 2C, D; $p = 1$). Although no difference was found in the PRs to sucrose plus strychnine and sucrose plus L-canavanine in both mode of presentations, we found a difference in the PR between *Gr66a>DTI* flies and their parents for sucrose plus L-canavanine (*Gr66a-Gal4*, $p = 0.007$; *UAS-DTI*, $p = 0.003$) in the mixed mode and only between one control strain and *Gr66a>DTI* for the dissociated presentation.

Because *Gr66a* is not expressed ubiquitously in taste neurons detecting bitter chemicals on the tarsi (Ling et al., 2014), we also tested ablating cells expressing *Gr33a*, which is expressed in bitter-sensitive cells (Moon et al., 2009; Lee et al., 2010). A

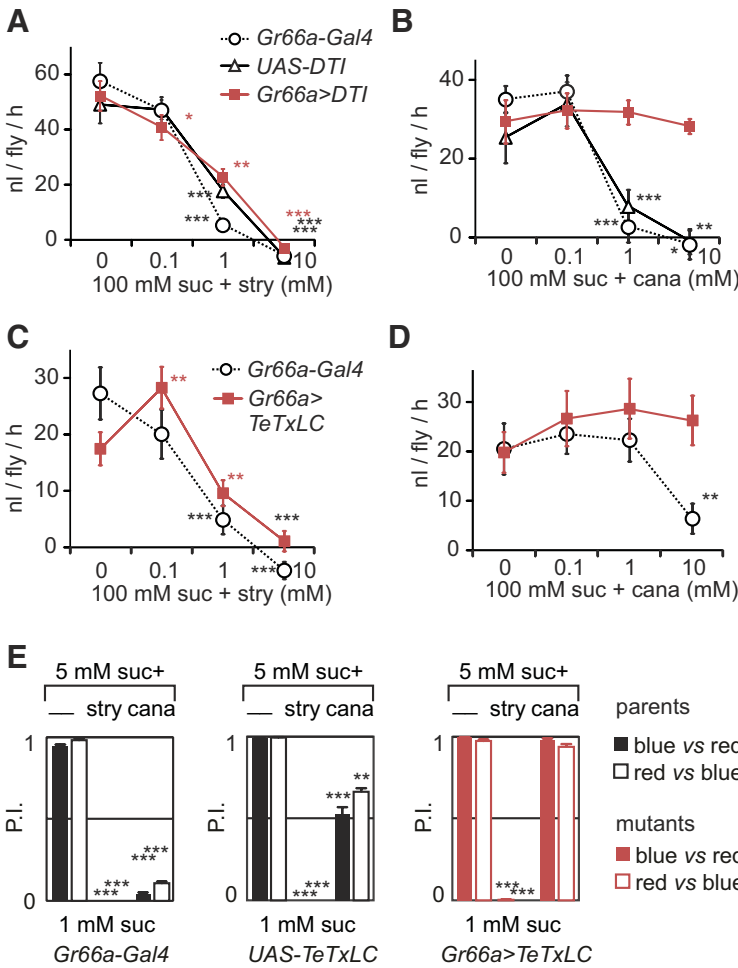


Figure 4. Flies with ablated bitter-sensitive cells are still able to avoid sucrose solutions containing strychnine in MultiCAFE and binary choice tests. **A–D**, Flies were given access to 5 μ l capillary tubes filled with 100 mM sucrose and different concentrations of strychnine or L-canavanine. We measured the consumption of groups of 20 flies during 2 h in the dark (nanoliters per fly per hour), displaying the results as mean \pm SEM. The differences between consecutive concentrations were determined using GLMM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; nonsignificant values are not reported on the graph). **A**, We observed a significant reduction of consumption according to strychnine concentration (0, 0.1, 1, and 10 mM) mixed with 100 mM sucrose in *Gr66a>DTI* flies (with bitter-sensitive cells ablated) and in their parental lines (*Gr66a-Gal4* and *UAS-DTI*) ($n = 13$ repetitions for each strain). **B**, We also observed a significant reduction of consumption according to L-canavanine (0, 0.1, 1, and 10 mM) mixed with 100 mM sucrose in the parental lines (*UAS-DTI*, $n = 8$; *Gr66a-Gal4*, $n = 12$) but not in *Gr66a>DTI* flies ($n = 13$). **C–E**, To confirm that the DTI could be replaced by another method of impairing cells expressing Gr66a, we used TeTxLC to selectively inactivate the synapses of these cells. **C**, We observed a significant reduction of consumption in response to strychnine mixed with sucrose in one parental line (*Gr66a-Gal4*, $n = 10$) and *Gr66a>DTI* flies ($n = 7$). **D**, We observed also a reduction of consumption in response to L-canavanine mixed with sucrose in the parental line tested (*Gr66a-Gal4*, $n = 10$) but not in *Gr66a>DTI* flies ($n = 10$). **E**, Flies expressing the TeTxLC were also tested in a two-choice test, in which groups of 80 flies were given access to agar wells filled with a red or blue food dye mixed with either 1 mM sucrose or 5 mM sucrose plus 30 mM L-canavanine or 10 mM strychnine. After feeding 2 h in the dark, we measured the proportion of flies with their abdomen colored in blue, red, or pink to compute a PI [$PI = (n_{blue} + n_{pink}/2)/(n_{blue} + n_{pink} + n_{red})$] and performed the same experiment after inverting the dyes (black bars, blue vs red; white bars, red vs blue; $n = 4$ repetitions for each condition). The statistical significance of the results was evaluated with unpaired Student's *t* tests with unequal variances. We observed a strong reduction of PI when sugar was mixed with strychnine or L-canavanine in the parental lines ($p < 0.0001$). In *Gr66a>TeTxLC* flies, we observed also a strong reduction of the consumption in the presence of strychnine ($p < 0.001$) but not with L-canavanine ($p = 0.19 - 0.13$). The data are represented as the mean \pm SEM using the same conventions as in other figures for indicating the statistical significance level. suc, Sucrose; cana, L-canavanine; nico, nicotine; caff, caffeine; lob, lobeline; stry, strychnine; den, denatonium.

difference in the PRs to sucrose plus L-canavanine between the control strains and *Gr33a>DTI* flies was observed (Fig. 2E,F) as in *Gr66a>DTI* flies. In addition, significant differences were observed in the PERs to sucrose plus strychnine in both presentation modes.

These observations suggest that taste cells expressing Gr66a and Gr33a play a role in triggering PRs (as found on the pro-

boscis) and also that Gr33a cells contribute to prevent PERs. However, in all strains tested here, the effect of strychnine was more potent on the PER when presented within a mixture than when presented separately, whereas the mode of presentation had no effect on the PR.

PERs/PRs to sugar and optogenetic activation of bitter-sensitive cells

To test whether activating bitter cells per se is sufficient to reduce sugar-induced PERs, we expressed ChR2 into Gr66a cells to activate them with light (Fig. 3A). In *Gr66a>ChR2* flies, we observed a 22% reduction of the PER by BL (Fisher's exact test, $p = 0.024$; Fig. 3A), although no effect was observed in the control strains (*Gr66a-Gal4* and *UAS-ChR2*; $p = 1.000$). We observed a significant increase of the PR in *Gr66a>ChR2* flies stimulated with sucrose plus BL compared with sucrose alone ($p = 0.011$; Fig. 3A), although no effect was detected in control flies ($p = 0.7445$).

Thus, the optogenetic activation of Gr66a cells of the legs and the proboscis exerts only a moderate inhibition of the PER but a marked effect on the PR, mimicking the effect of the dissociated presentation mode with strychnine (Fig. 2C,D) and of L-canavanine on responses on the labellum (Fig. 1B,C).

PERs/PRs to optogenetic activation of sugar-sensitive cells and strychnine

Conversely, we expressed ChR2 into Gr64f cells to mimic the effect of sugar with light (Fig. 3B). *Gr64f>ChR2* flies responded to BL stimulation with a robust PER, whereas flies from the control strains did not respond (Fig. 3B). We found an ~29% reduction in the PER when *Gr64f>ChR2* flies were stimulated with BL plus 10 mM strychnine compared with BL plus water (as control for the tactile stimulation; Fisher's exact test, $p = 0.0073$). No difference was observed in the PER when the control strains were stimulated with BL plus water and BL plus strychnine ($p = 0.695$). A significant increase in the PR was observed when *Gr64f>ChR2* flies were stimulated with BL plus strychnine compared with BL plus water alone ($p = 0.0036$).

In summary, the BL-induced PER is inhibited by strychnine to an extent comparable with the dissociated presentation (Fig. 2B).

Behavioral responses of flies to sugar/bitter solutions in MultiCAFE and two-choice tests

Our observations on PERs/PRs indicate that strychnine mixed with sucrose plays a stronger role than strychnine alone, whereas

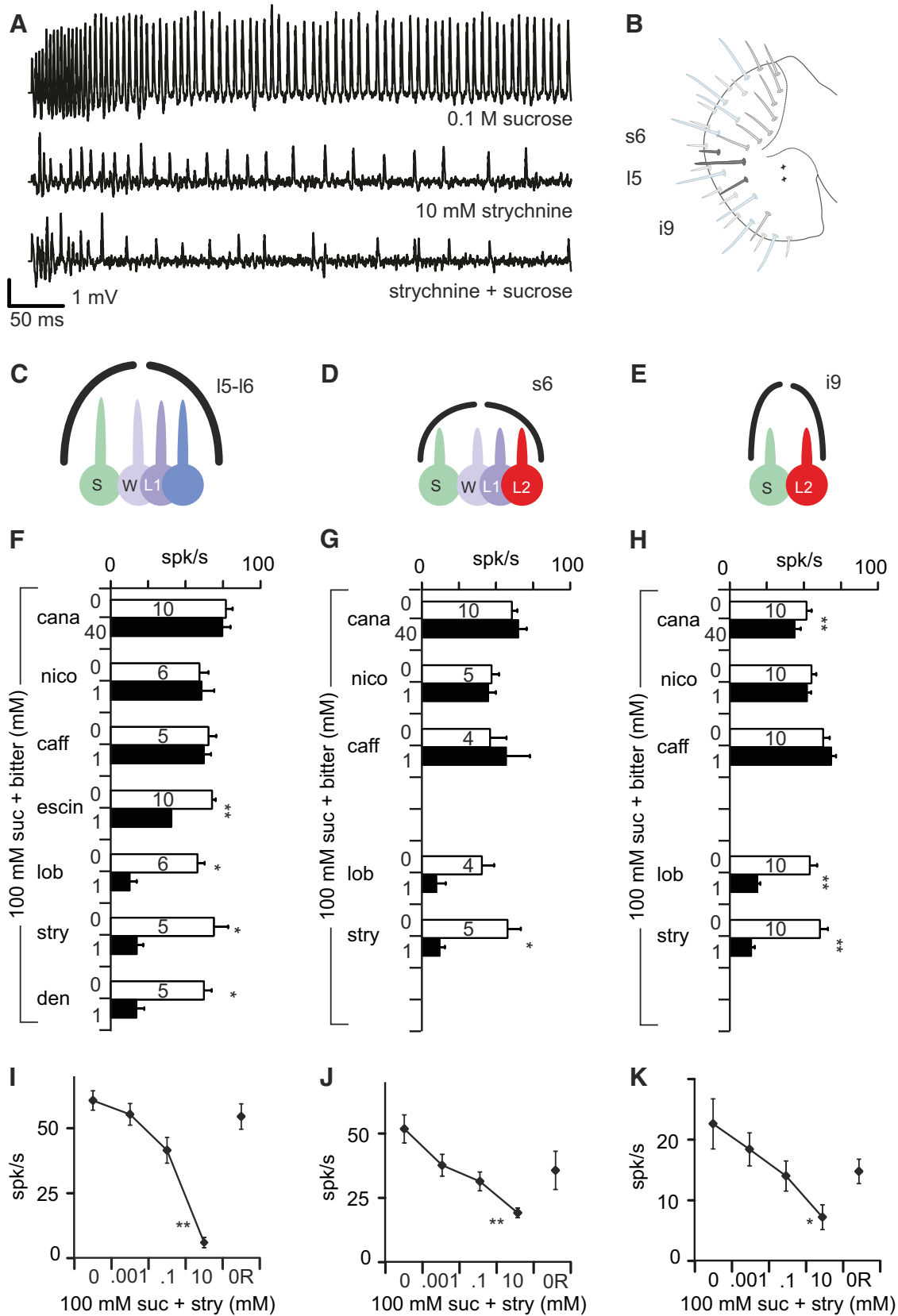


Figure 5. Bitter chemicals differ in their capacity to inhibit sucrose detection. **A**, Representative sample of strychnine-induced inhibition of the responses to sugar in taste sensilla obtained with a tip-recording electrode. Top trace shows the first 500 ms of a recording in which one neuron is firing vigorously when stimulated with 0.1 M sucrose (+ 100 mM TCC used as an electrolyte). The middle trace shows the activation of another neuron in response to 10 mM strychnine. The bottom trace shows the firing activity recorded in response to a mixture of these two stimuli with much less action potentials than expected if the two neurons would react to sugar and strychnine independently. **B**, Location of the taste sensilla mostly recorded from in this study, using a map notation introduced by Hiroi et al. (2002) and cellular composition of the sensilla tested I5 and I6 sensilla host four taste cells (**C**) and s6 sensilla (**D**), whereas i9 sensilla host only two taste cells (**E**). These taste cells are labeled according to their sensitivity as S (sugar-sensitive), W (water-sensitive), L1 (low salt-sensitive), and L2 (bitter-sensitive). Bitter chemicals (but not all) (*Figure legend continues.*)

whether L-canavanine is mixed or alone makes no difference. Because these observations were performed on a short timescale (within seconds), these differences may not affect feeding activities on a longer timescale, which are regulated by post-ingestive mechanisms. Insects are indeed capable of rejecting noxious substances through post-ingestive mechanisms (Glendinning, 1996; Wright et al., 2010) and to quickly learn the nutritive value of food (Burke and Waddell, 2011; Dus et al., 2011; Fujita and Tanimura, 2011; Gruber et al., 2013; Bjordal et al., 2014). Thus, we evaluated whether defects in peripheral detection of bitter molecules inherent in *Gr66a>DTI* flies would be compensated for in behavioral tests lasting several hours.

In MultiCAFE assays, *Gr66a>DTI* flies and their parental lines preferred feeding from capillary feeders without strychnine in relation to the dose ($p < 0.001$, GLMM; Fig. 4A). However, *Gr66a>DTI* flies did not discriminate between sugar solutions containing different concentrations of L-canavanine ($p = 0.967$; Fig. 4B), whereas the parental lines had no difficulty avoiding 1 and 10 mM L-canavanine mixed with sugar ($p < 0.001$, GLMM; Fig. 4B).

To confirm that these differences were not attributable to the diphtheria toxin, we silenced bitter-sensitive cells using *TeTxLC*. In a MultiCAFE assay, *Gr66a>TeTxLC* flies and one parental line preferred feeding from capillaries without strychnine in relation to the dose ($p < 0.001$, GLMM; Fig. 4C). However, *Gr66a>TeTxLC* flies did not discriminate between different solutions of L-canavanine ($p = 0.2088$; Fig. 4D), whereas one parental line avoided sucrose solutions containing 10 mM L-canavanine ($p < 0.0001$, GLMM; Fig. 4D).

We further tested these flies in a two-choice feeding assay. In the presence of strychnine, both parental lines and *Gr66a>TeTxLC* flies completely avoided the treated wells ($p < 0.001$, unpaired Student's *t* test; Fig. 4E). In contrast, *Gr66a>TeTxLC* flies were unable to detect L-canavanine (blue/red, $p = 0.191$; red/blue, 0.131). One parental line (*Gr66a-Gal4*) completely avoided L-canavanine ($p < 0.001$), whereas the other (*UAS-TeTxLC*) was less sensitive. This is likely attributable to a genetic background effect (blue/red, $p = 0.0001$; red/blue, $p < 0.001$).

Altogether, these results indicate that bitter cells expressing *Gr66a* are necessary to detect L-canavanine but are dispensable for detecting strychnine in both MultiCAFE and two-choice feeding assays, which assess feeding behavior over a 2 h timeframe.

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(Figure legend continued.) elicit spiking responses in i9 and s6 sensilla but not in l5 and l6 sensilla. **F–H**, Extracellular spiking activity recorded with sucrose mixed with different bitter chemicals recorded in *w¹¹¹⁸* flies in l5 (**F**), s6 (**G**), and i9 (**H**) sensilla. We compared the responses to 100 mM sucrose (suc; white bar) with the responses to 100 mM sucrose mixed with a bitter chemical (black bar). The molecules tested were L-canavanine (cana), nicotine (nico), caffeine (caff), escin, lobeline (lob), strychnine (stry), and denatonium (den). All molecules were at 1 mM, except 40 mM for L-canavanine. Data are displayed as the mean and SEM ($n = 4–10$ as reported on the bar of each graph). The difference between the response to sucrose (first presentation) and to the mixture was determined using Wilcoxon's matched pairs tests. **I–K**, Extracellular spiking activity in response to 100 mM sucrose mixed with different concentrations of strychnine in l6 (**I**), s6 (**J**), and i9 (**K**) sensilla. We measured the spiking activity (spikes not sorted) in response to 100 mM sucrose mixed with increasing concentrations of strychnine (0, 0.001, 0.1, and 10 mM). Sucrose alone was tested at the end of the experiment again to evaluate the response recovery (OR). $n = 5–6$ for each stimulation. Error bars represent SEM. The statistical significance was estimated with a Kruskal–Wallis test, with strychnine concentration as a categorical predictor. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Electrophysiological responses of labellar taste sensilla to sucrose mixed with different bitter molecules

To examine the cellular basis of these behavioral observations, we looked at the electrophysiological responses of taste sensilla stimulated with sucrose, with a bitter molecule, and with their mixture. As noted in previous studies (Meunier et al., 2003a; Jeong et al., 2013), whereas sucrose activates a cell and strychnine activates another cell, when the two chemicals are mixed, the resulting response is much less than expected by simply summing the activity of the two cells to each chemical (Fig. 5A).

We examined taste sensilla of the proboscis (Fig. 5B) that are mapped and identified according to their length and position (Shanbhag et al., 2001; Hiroi et al., 2002). Short-type sensilla (s-type) and long-type sensilla (l-type) house four taste neurons, whereas intermediate-sized (i-type) sensilla house only two taste neurons. In s-type sensilla, each taste neuron responds to different groups of molecules: sugars (Hiroi et al., 2002, 2004), water (Cameron et al., 2010; Chen et al., 2010), salts (Meunier et al., 2003b) or pheromones (Thistle et al., 2012), and bitter chemicals (Hiroi et al., 2002; Dahanukar et al., 2007; Weiss et al., 2011; Fig. 5C–E, cells S, W, L1, and L2, respectively). In l-type sensilla, three of these cells are present, but none is responding to bitter chemicals (Hiroi et al., 2002; Weiss et al., 2011). In i-type sensilla, one neuron responds to sugars whereas the other responds to bitter molecules (Hiroi et al., 2004; Fig. 5E, cells S and L2, respectively).

First, we examined whether all bitter chemicals would inhibit sugar-sensitive cells by recording the responses of taste sensilla l5–l6, s6, and i9 to sucrose, and then to sucrose mixed with a bitter molecule (Fig. 5F–H). An additional stimulation with sucrose was made to ensure that cells were not intoxicated. Sensilla for which the response to the third stimulation with sucrose was suppressed were discarded. This was to ensure that any observed reduction in response to sucrose when mixed with bitter molecules was attributable to a sensory inhibition rather than an enduring toxic effect. Unless otherwise noted in this series of measurements and in all other experiments, we report on the total number of spikes detected during the first second of each recording. The spikes were not sorted and thus could represent the activity of several neurons.

In l5–l6 sensilla, we observed a reduction of activity when 100 mM sucrose was mixed with 1 mM lobeline, strychnine, denatonium, or escin (Wilcoxon's paired test, $p = 0.005$, 0.028, 0.043, and 0.043; $n = 10$, 6, 5, and 5 respectively; Fig. 5F). We did not observe a statistically significant reduction of activity with 1 mM caffeine, 1 mM nicotine, or 40 mM L-canavanine ($n = 10$, 6, and 5, respectively; Fig. 5F). Similar results were obtained with s6 sensilla ($n = 4–10$), in which only strychnine was found to inhibit sugar detection to a statistically significant level ($p = 0.04$, $n = 5$; Fig. 5G). In i9 sensilla ($n = 10$), L-canavanine, strychnine, and lobeline were found to inhibit sugar detection ($p = 0.008$, 0.005, and 0.005, respectively; Fig. 5H).

We further examined the effect of increasing concentrations of strychnine on the response to 100 mM sucrose in these sensilla (Fig. 5I–K). We observed a consistent reduction in response to sucrose when mixed with 10 mM strychnine in l6 (Kruskal–Wallis test, $p = 0.001$), i9 ($p = 0.04$), and s6 sensilla ($p = 0.003$). In all three sensilla, the response to sucrose at the end of the experiment was not statistically different from the response to sucrose at the beginning of the series ($p = 1.000$).

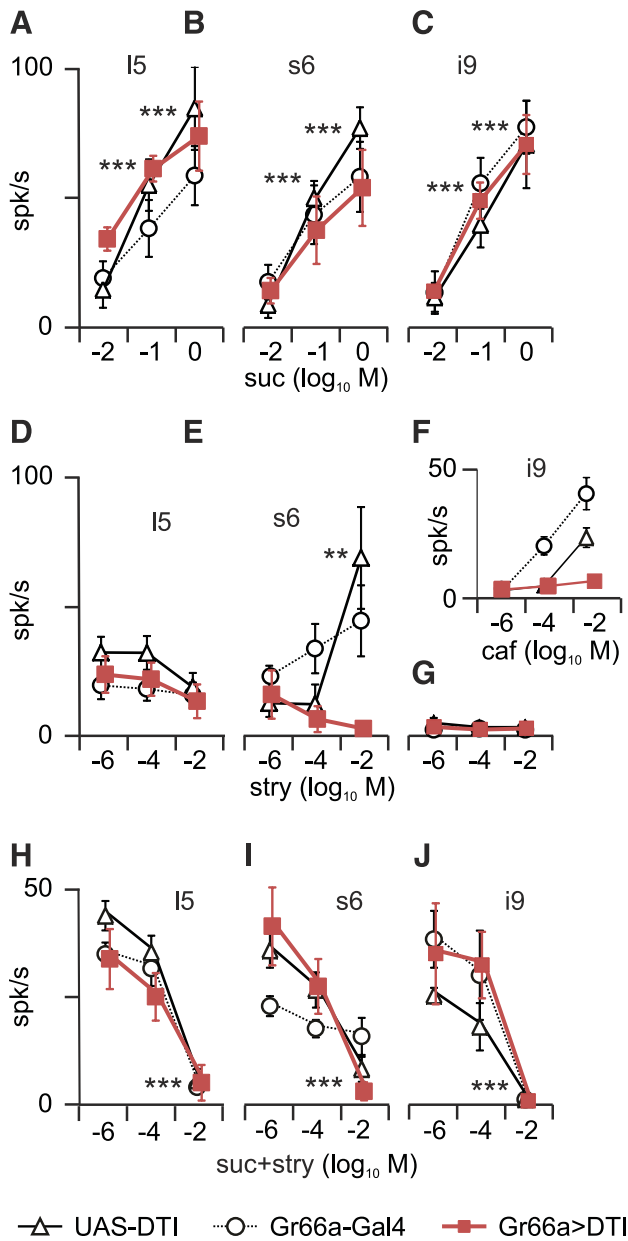


Figure 6. Strychnine inhibition of sucrose responses persists in labellar s6, i9, and I5 sensilla of *Gr66a>DTI* flies. Extracellular responses recorded from sensilla on the labellum (mean \pm SEM) in s6 (left), I5 (middle), and i9 (right) sensilla in *Gr66a>DTI* (red squares) and the parental lines (triangle, *UAS-DTI*; circle, *Gr66a-Gal4*). **A–C**, Responses to 0.01, 0.1 and 1 mM sucrose were dose dependent in all strains in s6 sensilla (**A**; *Gr66a>DTI*, $n = 6$; *Gr66a-Gal4*, $n = 5$; *UAS-DTI*, $n = 5$), I5 sensilla (**B**; $n = 7, 6, 5$, respectively), and i9 sensilla (**C**; $n = 11, 6, 5$, respectively). **D–G**, Responses to 0.001, 0.1, and 10 mM strychnine were reduced in s6 sensilla (**D**) of *Gr66a>DTI* flies ($n = 7, 6, 5$, respectively) but not in I5 sensilla (**E**) that are not activated in response to bitter chemicals ($n = 6, 5, 6$, respectively) and in i9 sensilla (**G**) that are not sensitive to strychnine ($n = 10, 5, 5$, respectively). **F**, To check that the bitter-sensitive neuron was ablated in i9 sensilla, we tested the responses to 0.001, 0.1, and 10 mM caffeine ($n = 11, 5, 5$, respectively). **H–J**, Responses to a mixture of 0.1 mM sucrose and 0.001, 0.1, and 10 mM strychnine in s6 sensilla (**H**; $n = 6, 6, 5$, respectively), I5 sensilla (**I**; $n = 7, 6, 5$, respectively) and i9 sensilla (**J**; $n = 11, 5, 5$, respectively). A dose-dependent effect of strychnine was found in all three sensilla in the three strains. These data were analyzed with a GLIMM procedure. Abscissa, Log₁₀ of the molar concentration. *** $p < 0.01$, **** $p < 0.001$. suc, Sucrose; stry, strychnine; caf, caffeine.

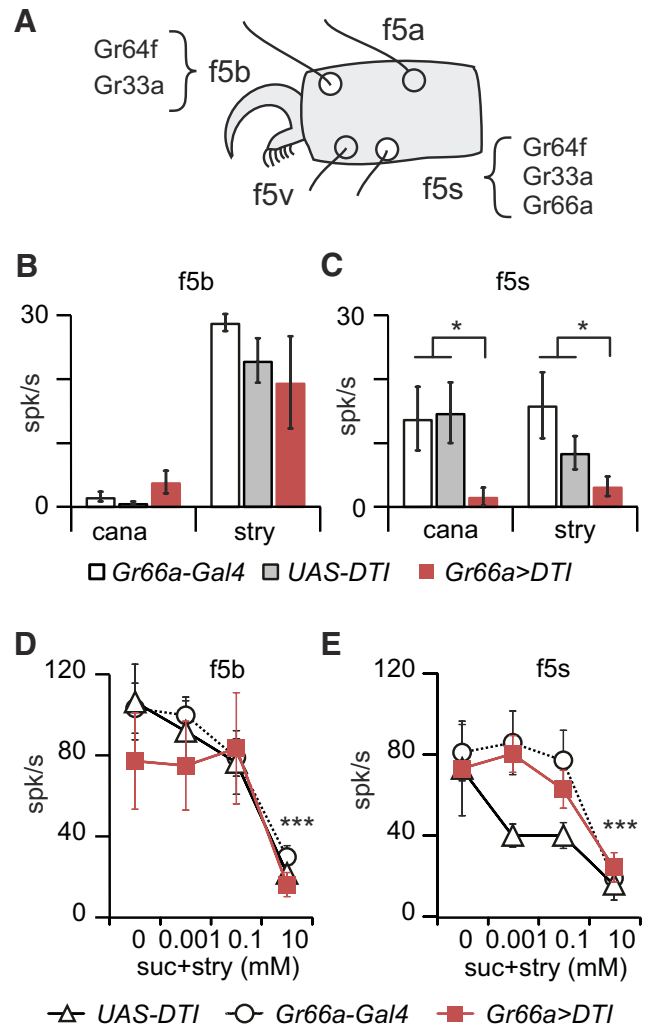


Figure 7. Strychnine inhibition of the responses to sucrose persists in tarsal f5b and f5s sensilla of *Gr66a>DTI* flies. **A**, Schematic drawing of the position of f5b and f5s sensilla on the fifth tarsus of female flies. According to Ling et al. (2014), only f5s express Gr66a, which means that f5b should remain functional in *Gr66a>DTI* flies. **B, C**, Extracellular spiking activities (mean \pm SEM) recorded in responses to strychnine and L-canavanine in *Gr66a>DTI* (red bars), *UAS-DTI* (gray bars), and *Gr66a-Gal4* (white bars) flies. **B**, In f5b sensilla, the responses of the three strains are equivalent with respect to 10 mM strychnine (*Gr66a>DTI* vs *UAS-DTI*, $p = 0.55$ and *Gr66a>DTI* vs *Gr66a-Gal4*, $p = 0.50$, $n = 4-7$; Kruskal–Wallis test) and 40 mM L-canavanine (*Gr66a>DTI* vs *UAS-DTI*, $p = 1.00$ and *Gr66a>DTI* vs *Gr66a-Gal4*, $p = 0.76$, $n = 4-8$). **C**, In f5s sensilla, we found a significant difference in the responses to 10 mM strychnine between *Gr66a>DTI* and the parental lines (*Gr66a>DTI* vs *Gr66a-Gal4* + *UAS-DTI*, $p = 0.011$, $n = 4-6$) and 40 mM L-canavanine (*Gr66a>DTI* vs *Gr66a-Gal4* + *UAS-DTI*, $p = 0.015$, $n = 3-7$). **D, E**, We further tested the responses to 0.1 mM sucrose mixed with strychnine (0, 0.001, 0.1, and 10 mM) in *Gr66a>DTI* (red squares), *UAS-DTI* (white triangle), and *Gr66a-Gal4* (white circle) flies. In f5b sensilla (**D**) and f5s sensilla (**E**) in the three genotypes ($n = 4-10$) in which we found a significant effect of concentration in f5b sensilla ($p < 0.001$, GLMM procedure) and f5s sensilla ($p = 0.0011$, GLMM). * $p < 0.05$, **** $p < 0.001$. cana, L-Canavanine; stry, strychnine; suc, sucrose.

Electrophysiological responses of labellar taste sensilla of *Gr66a>DTI* flies

Our behavioral observations together with our initial electrophysiological recordings suggest that strychnine inhibits sugar-detection cells and activates bitter-sensitive cells, whereas L-canavanine mostly activates bitter-sensitive cells. To determine whether bitter-sensitive neurons are required for sugar-sensitive neuron inhibition, we recorded the responses of flies in which cells expressing Gr66a are ablated, using the same genetic construction used in

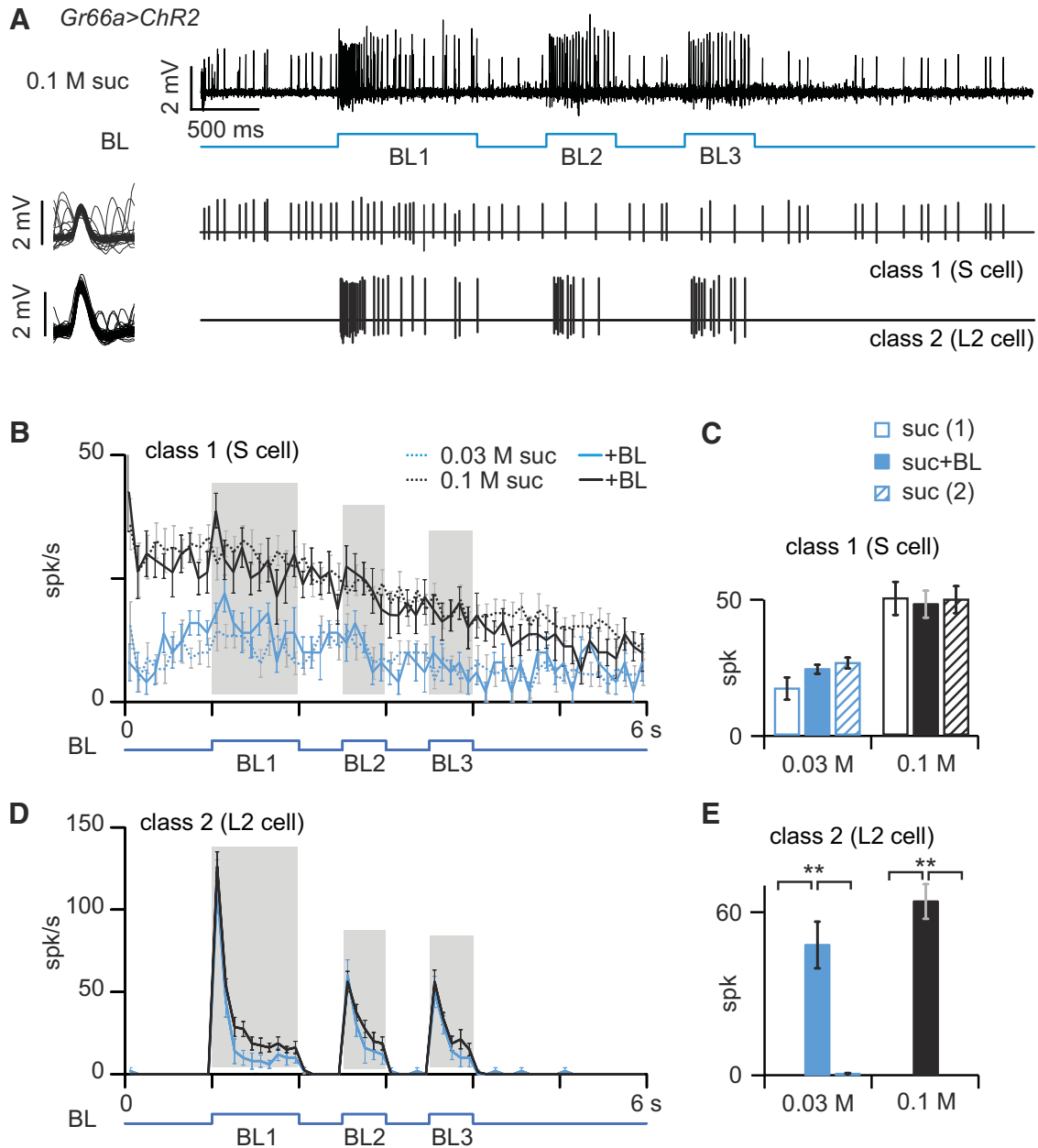


Figure 8. Optogenetic activation of the bitter-sensing cell does not inhibit responses to sugar. **A**, Labellar sensilla of *Gr66a>ChR2* flies were stimulated with sucrose (suc) and BL (3 bursts of 1 s, BL1; 0.5 s, BL2 and BL3). The recordings were performed in i9 sensilla in which two classes of spikes can be separated on the basis of their amplitude, shape, and temporal occurrence. The spikes extracted from the sample recording (top trace) are displayed below as a succession of bars at the same timescale and on the left by superimposing each spike of the series in a 6 ms window. Small spikes corresponded to the activity of a cell responding to sucrose, called S. Larger-amplitude spikes corresponded to the activity of a neuron responding to BL (and to bitter chemicals), called L2. **B**, Spiking activity of S-cells in response to sucrose at 2 concentrations (0.1 M, black curves; 0.03 M, blue) with or without optogenetic stimulation (continuous and dotted lines, respectively). Each point of the curve represents the number of spikes in 100 ms bins (mean \pm SEM; $n = 5–15$ recordings). The number of spikes occurring during the three intervals BL1, BL2, and BL3 were added to compare the activity of the neuron in the presence or absence of BL. **C**, Histogram of the number of spikes recorded (mean \pm SEM; $n = 3–9$) during the presentation of BL (BL1 + BL2 + BL3, shaded area in **B**). Each sensilla were stimulated with sucrose only before [suc(1), open bars] and after [suc(2), hatched bars] the optogenetic stimulation (suc + BL, filled bar). Sucrose was presented at 2 concentrations: 0.03 M (blue bars) and 0.1 M (black bars). The statistical significance of the differences between the responses in the three situations were evaluated with a Kruskal–Wallis ANOVA. **D**, Spiking activity of L2 cells extracted from the same recordings displayed in **B**. **E**, Histograms of the responses of L2 cells in the same recordings as in **C**. $**p < 0.01$.

the behavioral experiments (*Gr66a>DTI*), in sensilla with four taste neurons (I5, s6), and with two neurons (i9; Fig. 5C–E).

With sucrose (Fig. 6A–C), we observed a dose-dependent response in *Gr66a>DTI* flies and their parental lines in all sensilla tested ($p < 0.0001$, GLMM). With strychnine (Fig. 6D,E,G), a dose-dependent response was found in the parental lines in s6 sensilla ($p < 0.001$, GLMM) but not in I5 or i9 sensilla, in which strychnine had been documented to elicit very low responses

(Weiss et al., 2011). In *Gr66a>DTI* flies, the responses to strychnine was suppressed in s6 sensilla ($p = 0.12$, GLMM). To check whether i9 sensilla were responsive to bitter chemicals, we tested them with caffeine (Fig. 6F).

In response to mixtures of 0.1 M sucrose and strychnine (Fig. 6H–J), we found a reduction of the spiking activity in all sensilla, in the control strains and *Gr66a>DTI* flies ($p < 0.001$, GLMM).

These observations confirm that sugar-induced inhibition is present in sensilla that are missing cells activated by strychnine either naturally as in I-type sensilla or when bitter-sensitive cells (expressing Gr66a) are ablated.

Electrophysiological responses of leg taste sensilla of *Gr66a/DTI* flies

We also recorded from taste sensilla on the legs, first, to confirm that strychnine inhibits sugar detection and, second, to further establish that some bitter-sensitive neurons are not ablated on the legs in *Gr66a>DTI* flies. We selected sensilla f5b and f5s (Fig. 7A) because Gr66a is expressed only in f5s, whereas Gr33a is expressed in both (Ling et al., 2014). Therefore, we expected to find in *Gr66a>DTI* flies a suppression of the responses to strychnine and L-canavanine in f5s but not in f5b.

First, we checked that these sensilla responded to L-canavanine and strychnine and reacted differently to the ablation of Gr66a cells (Fig. 7B,C). The response to strychnine and to L-canavanine was significantly different in f5s sensilla in *Gr66a>DTI* flies compared with the parental lines (*Gr66a>DTI* vs *Gr66a-Gal4 + UAS-DTI*; strychnine, $p = 0.011$; L-canavanine, $p = 0.015$, Kruskal–Wallis test; Fig. 7C) but not in f5b sensilla (strychnine, $p = 0.558$ for *Gr66a-Gal4* and $p = 1.000$ for *UAS-DTI*; no responses were recorded with L-canavanine; Fig. 7B).

Then, we looked at the response to mixtures of strychnine with sucrose (Fig. 7D,E). Strychnine concentration had a highly significant effect in all strains because it inhibited sugar detection in both the parental lines and *Gr66a>DTI* flies in f5b and f5s sensilla ($p < 0.0001$, GLMM).

These data confirm that strychnine inhibits sugar detection in sensilla other than on the labellum and that the construction *Gr66a>DTI* does not completely abolish the detection of strychnine in all sensilla of the tarsus because f5b sensilla keep their sensitivity toward strychnine (Fig. 7B).

Electrophysiological responses of taste sensilla stimulated with sucrose during optogenetic activation of taste cells expressing Gr66a

Although our previous observations indicate that bitter-sensitive cells are not necessary for inhibiting the detection of sugars, it does not rule out the possibility that activating a bitter-sensing cell could reduce firing in adjacent sugar-sensitive neurons. Such a mechanism has been demonstrated recently in the olfactory system of *Drosophila*, in which the transient activation of an olfactory receptor neuron (ORN) can inhibit the sustained activity of a neighboring ORN (Su et al., 2012).

We asked whether optogenetic activation of bitter-sensing cells could inhibit the response to sucrose in i9/i8 sensilla of *Gr66a>ChR2* flies. In the absence of optogenetic activation, sucrose induced a tonic response in sugar-sensitive cells (Fig. 8B) but not in bitter-sensitive cells (Fig. 8D). In the presence of light, bitter-sensitive cells displayed a phasic–tonic excitation (Fig. 8A,D,E) that did not affect the time course of the responses to sugar in the sugar-sensitive cells (Fig. 8B–D).

Electrophysiological responses of sugar-sensitive taste neurons activated optogenetically in the presence of strychnine

We then asked whether strychnine inhibits sugar-induced responses by interfering with general cellular excitation or whether this inhibition is specific to sugar transduction. To address this question, we used *Gr64f>ChR2* flies. If strychnine induces a gen-

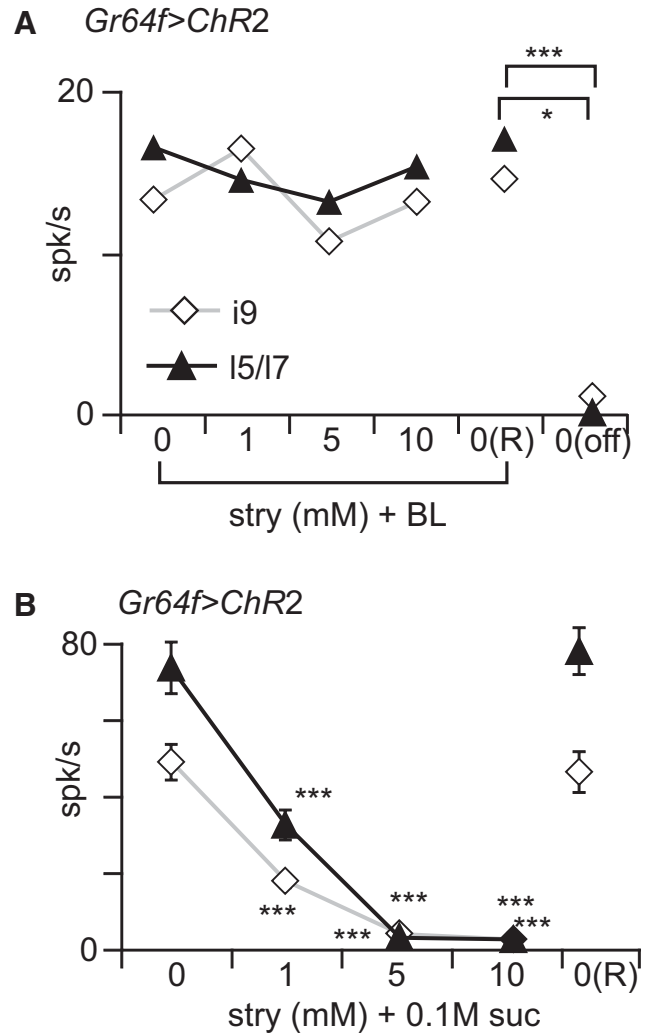


Figure 9. Strychnine does not inhibit responses induced by ectopic ChR2. **A**, *Gr64f>ChR2* flies were stimulated with a 2 s pulse of BL and concurrently with different concentrations of strychnine. We analyzed the number of spikes (mean \pm SEM) during the first second of the recordings in i9 (white diamonds; $n = 7$) and I5–I7 (black triangles; $n = 11$) sensilla in the presence of different concentrations of strychnine delivered sequentially in an increasing order of concentrations (0, 1, 5, and 10 mM), followed by BL only [0(R)] and nothing [0(off)]. These data were analyzed with a one-way ANOVA with Fisher's *post hoc* analysis. No effect of strychnine concentration on the response to BL was observed. Significant differences between 0(off) and 0, 1, 5, 10, and 0(R) were observed for i9 and I5/I7; however, only the interaction between 0(off) and 0 is shown on graph. **B**, As a control, we verified that *Gr64f>ChR2* flies respond to sugar and that these responses are inhibited by strychnine. Black triangles, I5–I7 sensilla ($n = 11$); white diamonds, i9 sensilla ($n = 9$). stry, Strychnine; suc, sucrose; * $p < 0.05$; *** $p < 0.001$.

eral inhibition of the sugar-sensing neurons, we would expect it to reduce the response of *Gr64f>ChR2* neurons to BL.

We stimulated i9 and I5–I7 sensilla with BL and strychnine (Fig. 9A). No significant effect of strychnine concentration on BL response was observed (one-way ANOVA, $p = 0.79$ for I5–I7 and $p = 0.813$ for i9). We found a significant difference between the response of I5–I7 and i9 when stimulated with TCC in the presence and absence of BL (Fisher's LSD test, $p < 0.001$ for i9 and $p < 0.001$ for I5–I7). However, in these flies, strychnine inhibited responses to sucrose (Kruskal–Wallis ANOVA by ranks, $p < 0.001$; Fig. 9B). We infer from this that strychnine does not inhibit sugar-sensing cells but that it directly interferes with sugar-specific reception or transduction pathways.

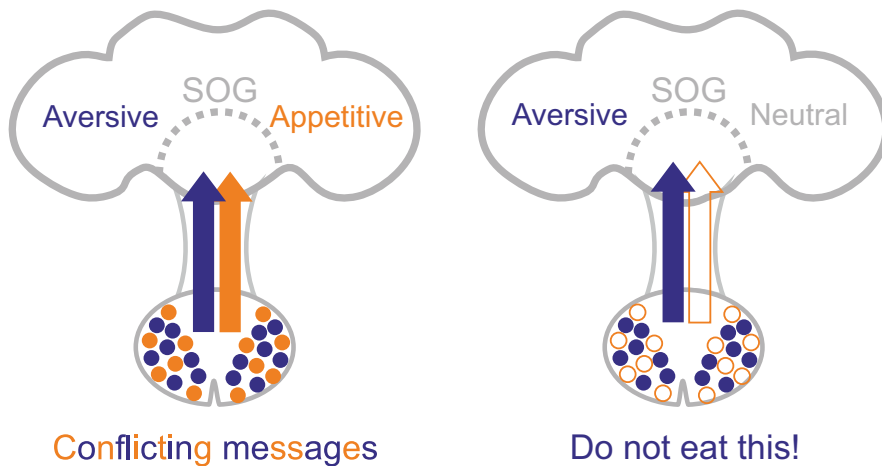


Figure 10. Sugar-sensing inhibition simplifies the processing of conflicting messages. Sweet molecules alone are activating S cells (orange dot) that express sugar receptors such as *Gr64f*. Bitter molecules alone are activating L2 cells (blue dots) that express *Gr66a* on the labellum. These neurons project in separate areas in the subesophageal ganglion (SOG). In the presence of a mixture of sugar and bitter molecules, if these two detection channels were independent, one would expect the two populations of cells to be activated simultaneously. Our data show that bitter molecules inhibit the detection of sugar molecules. This suggests that, in flies, the detection of bitter molecules within mixtures is encoded in two ways: (1) activating bitter-sensitive cells; and (2) inhibiting sugar sensing through the sugar-sensitive cells, thereby making appetitive stimuli less attractive when mixed with bitter substances.

Discussion

In this work, we evaluated the respective roles of two pathways contributing to the detection of bitter compounds, the activation of bitter-sensitive cells, and the inhibition of sugar detection. The importance of inhibiting sugar detection is exemplified by the observation that flies deprived of bitter-sensitive cells retain the capacity to avoid feeding from sugar solutions containing strychnine almost as well as normal flies. Although the activation of bitter-sensitive cells induce active aversive reactions, such as the PR, sugar-sensing inhibition is very effective in preventing feeding from appetitive solutions when spiked with bitter chemicals. We established that chemicals such as strychnine, lobeline, denatonium, and eschin inhibit sugar detection, whereas other chemicals such as L-canavanine, caffeine, and nicotine were not effective at the concentration tested. Given the importance of sugar-sensing inhibition, these observations are consistent with former behavioral observations showing that strychnine and lobeline were more potent anti-feedants than caffeine and nicotine in MultiCAFE (Sellier et al., 2011), two-choice assays (Meunier et al., 2003a; Weiss et al., 2011), and a visit-frequency assay (Marella et al., 2006). We further established that mixture suppression by strychnine is dose dependent and reversible and affects sugar detection in each type of sensilla on the proboscis and the legs and that this inhibition is a process affecting specifically the sugar transduction.

Inhibition of sugar detection by bitter chemicals has been described in several animals, including insects, but the extent of this suppression and its role in the feeding behavior has been less characterized. A number of studies described mixture interactions at the periphery using electrophysiology in vertebrates (Formaker et al., 1997; Keast and Breslin, 2002; Frank et al., 2003, 2005; Green et al., 2010) and insects (Chapman, 2003; Meunier et al., 2003a; Moon et al., 2009; Lee et al., 2010; Sellier et al., 2011). The cellular and molecular mechanisms involved in this inhibition have remained elusive so far. Bitter chemicals may directly interact competitively with sugar receptors or with allosteric sites located on them (Xu et al., 2004; Galindo-Cuspinera et al., 2006; Milligan and Smith, 2007; Maillet et al., 2009; Imada et al., 2010) or via direct interaction with transduction cascade elements

(Naim et al., 1994; Talavera et al., 2008) through a rapid entry of amphipathic molecules into taste cells (Peri et al., 2000). In addition, the activation of bitter-sensing cells could laterally inhibit sugar-sensitive cells through neuromodulation as in vertebrates (Roper, 2006; Cao et al., 2009; Dando and Roper, 2009; Herneess and Zhao, 2009; Yarmolinsky et al., 2009) or through ephaptic interactions as found recently in insect olfactory sensilla (Su et al., 2012).

Our data establish that sugar-sensing cell inhibition is independent of bitter-cell activation and that this inhibition is specific to sugar detection. First, sugar responses are inhibited by strychnine in all sensilla of the proboscis independently of the presence of bitter-sensing cells in those sensilla. This is the case for I-type sensilla that are not equipped with a bitter-sensitive cell and for I9 sensilla that house only two chemosensory cells, in which the bitter-sensitive cell can be ablated genetically by expressing a toxin in

it. Furthermore, if bitter-sensing cells are activated by BL (using ChR2 ectopically expressed in Gr66a cells), the response to sucrose is not affected. This indicates that sugar inhibition can occur in the absence of bitter cells.

Second, sugar-sensitive cells are inhibited by molecules different from those that stimulate bitter-sensitive cells. Strychnine and lobeline (as well as quinine; Sellier et al., 2011) are very potent inhibitors, whereas L-canavanine, caffeine, and nicotine are less effective. This confirms that bitter activation and sugar inhibition are two separate mechanisms, in agreement with the observation that bitter-sensitive gustatory receptors are not expressed in sugar-sensitive cells (Thorne et al., 2004; Marella et al., 2006; Hiroi et al., 2008; Weiss et al., 2011).

Third, when sugar-sensitive cells are activated by BL (using ChR2 ectopically expressed in Gr64f cells), strychnine does not inhibit the response to BL. This indicates that strychnine does not depress the excitability of sugar-sensing cells and suggests that it interferes specifically with sugar reception or transduction pathways. Thus, our data suggest that sugar-sensing cells are equipped with transduction pathways sensitive to bitter chemicals.

Recently, it has been demonstrated that an OBP, OBP49a, is required for sugar inhibition by bitter chemicals (Jeong et al., 2013). OBP49a is expressed by an accessory cell of most gustatory sensilla and is secreted in the sensillum lymph. Its loss results in reduced sugar inhibition or avoidance behavior in the presence of bitter chemicals. OBP49a directly binds quinine and denatonium, both of which are sweet taste inhibitors. Biochemical and genetic evidences show that OBP49a becomes closely associated with the sugar receptor Gr64a, indicating that OBPs may bind bitters and bring them to the immediate proximity of sugar gustatory receptors (Jeong et al., 2013). The presence of OBPs may serve as a mechanism to amplify the sensitivity of sugar neurons by chaperoning the interaction. The authors suggest an alternative mechanism analogous to the OBP LUSH, which activates a pheromone receptor (OR67d) when loaded with the ligand *cis*-vaccenyl acetate (cVA; Laughlin et al., 2008). However, a recent report suggests that cVA induces olfactory receptor activity in the absence of LUSH (Gomez-Diaz et al., 2013). Our study and findings on gusta-

tory receptors by Jeong et al. (2013) raise the intriguing possibility that bitter chemicals directly interact with sugar gustatory receptors.

Although sugar-sensing inhibition is an intriguing property of sugar-detecting cells, it also plays a decisive role in allowing flies to avoid mixtures spiked with bitter chemicals. This was very clear when comparing the feeding responses of flies given access to sugar mixed with either strychnine or L-canavanine. Although L-canavanine detection is completely suppressed after *Gr66a*-cell ablation, strychnine is still detected in these flies. These observations suggest that sugar-sensing inhibition plays a major role in most feeding behavior paradigms used to test feeding activities in flies, including the PER.

PER experiments on flies in which *Gr66a* cells were ablated or that were expressing *ChR2* has allowed us to better understand the interplay of bitter detection and mixture suppression. Control strains, *UAS-DTI*, and *Gr66a-Gal4* flies extend their proboscis less frequently in response to strychnine mixed with sucrose than when strychnine and sucrose were presented on separate legs. *Gr66a>DTI* flies avoid extending their proboscis in the presence of strychnine and are impaired in retracting their proboscis. L-Canavanine does not inhibit the PER but triggers a subsequent PR that disappears in *Gr66a*-ablated flies.

In *Gr66a>ChR2* flies, the PER is reduced by ~22% when flies are dually stimulated with sucrose and BL compared with sucrose alone. This level of inhibition is comparable with the responses of flies presented with sucrose and strychnine in the dissociated PER paradigm. The activation of bitter-sensing cells through remote activation or by stimulating with strychnine in *Gr66a>ChR2* or *Gr64f>ChR2* flies, respectively, triggers the PR (Fig. 4). Our observations are consistent with the hypothesis that the PER is strongly modulated by sugar-sensing inhibition, whereas the PR is triggered through the activation of bitter-sensing cells.

We postulate that sugar inhibition may contribute to adaptation of insects to their environment and should be subjected to selection pressure. One possibility is that sugar-sensing inhibition could be associated with bitter molecules that are particularly toxic for the animals because this mechanism seems to be hardwired, whereas the aversion to bitter chemicals that are detected only by bitter-sensitive cells could be modulated at the level of the synapses by a variety of mechanisms. A partial support to this hypothesis comes from the observation that camphor aversion can be modulated whereas quinine aversion is not in relation to the relative toxicity of these compounds (Zhang et al., 2013).

We speculate that different adaptation strategies may exist across insect species, i.e., the same molecule could activate bitter cells and/or inhibit sugar cells differently. Alkaloids such as strychnine inhibits the detection of sugars in Lepidoptera larvae (Schoonhoven and van Loon, 2002) and sugar alcohols (but not sucrose) in *Lymantria dispar* (Martin and Shields, 2012). Contrary to *Drosophila*, the grasshopper *Schistocerca americana* does not detect L-canavanine by excitation but only by sugar-sensing inhibition (Chapman et al., 1991). In honeybees, sugar-sensing inhibition might be even more developed than bitter detection (de Brito Sanchez, 2011) because honeybees possess very few gustatory receptors.

In summary, our results suggest strongly that detection of noxious compounds involves at least two independent mechanisms: (1) the activation of bitter-sensitive cells; and (2) mixture suppression within sugar-sensitive cells. This inactivation mechanism may contribute to simplify the processing of messages sent to the brain by the taste receptors when confronted with conflicting messages (Fig. 10). Our observations do not challenge the view that taste coding involves labeled lines, but they should cer-

tainly encourage us to revise our view of the sensory space encoded by each taste quality.

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C. Article 3 : Sugars suppress bitter taste in *Drosophila*

(en préparation)

Abstract

Bitter taste is not only useful to organisms to avoid ingesting noxious molecules, but it is also a limitation as it could prevent organisms to feed on nutritious food. While animals can overcome distaste by central mechanisms, bitter taste suppression can also occur directly at the periphery, within gustatory receptor neurons. In this work, we evaluated if 12 sugars could reduce the response of bitter-sensitive cells in flies. We first checked if flies could overcome the aversive taste of 4 bitter substances (denatonium, berberine, umbelliferone, caffeine) when mixed with a panel of 12 sugars, including nutritious and non-nutritious molecules. We observed that flies consume more of the bitter solutions with increasing concentrations of sugar. We then looked at the responses of taste sensilla on the proboscis of these flies, and observed that the responses to mixtures of bitter and sweet substances were lower than the responses to the isolated compounds. By separating the respective activities of the bitter- and of the sugar-sensitive gustatory neurons, we showed that sugars and bitter molecules have reciprocal inhibitory effects. While the inhibition of the bitter sensitive responses were about 25 % at the maximal concentration tested, the responses to sugars were diminished up to 60%. We confirmed these observations by recording from gustatory sensilla in which the sugar-sensitive neurons had been ablated, by means of a genetic construction. We show that these flies exhibit similar sweet inhibition than in the control flies. Lastly, we show here that a mixture of two sugars is more efficient than a sugar alone. These observations demonstrate that sweet inhibition also occur in flies and suggest that this inhibition properties are due to the gustatory neuron itself rather than due to interactions between gustatory neurons within the same sensilla.

Sugars suppress bitter taste in *Drosophila*

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Abstract

Bitter taste is not only useful to organisms to avoid ingesting noxious molecules, but it is also a limitation as it could prevent organisms to feed on nutritious food. While animals can overcome distaste by central mechanisms, bitter taste suppression can also occur directly at the periphery, within gustatory receptor neurons. In this work, we evaluated if 12 sugars could reduce the response of bitter-sensitive cells in flies. We first checked if flies could overcome the aversive taste of 4 bitter substances (denatonium, berberine, umbelliferone, caffeine) when mixed with a panel of 12 sugars, including nutritious and non-nutritious molecules. We observed that flies consume more of the bitter solutions with increasing concentrations of sugar. We then looked at the responses of taste sensilla on the proboscis of these flies, and observed that the responses to mixtures of bitter and sweet substances were lower than the responses to the isolated compounds. By separating the respective activities of the bitter- and of the sugar-sensitive gustatory neurons, we showed that sugars and bitter molecules have reciprocal inhibitory effects. While the inhibition of the bitter sensitive responses were about 25 % at the maximal concentration tested, the responses to sugars were diminished up to 60%. We confirmed these observations by recording from gustatory sensilla in which the sugar-sensitive neurons had been ablated, by means of a genetic construction. We show that these flies exhibit similar sweet inhibition than in the control flies. Lastly, we show here that a mixture of two sugars is more efficient than a sugar alone. These observations demonstrate that sweet inhibition also occur in flies and suggest that this inhibition properties are due to the gustatory neuron itself rather than due to interactions between gustatory neurons within the same sensilla.

Introduction

The sense of taste is crucial to quickly evaluate if food resources might be nutritious and if they contain noxious chemicals (Lindemann 2001; Yarmolinsky *et al.* 2009). This sensory system must deal with numerous molecules produced by plants, many of which are toxic (Ames *et al.* 1990). Most if not all plant secondary compounds such as alkaloids, plant-based phenols, flavonoids, isoflavones, terpenes, and glucosinolates taste bitter, acrid, or astringent and are aversive to the consumer (Drewnowski and Rock 1995; Bravo 1998). Although in few cases, bitter taste is tolerated or acceptable e.g. tea, coffee, wine, beer or bitter lemon (Ley *et al.* 2005), the bitterness of legumes and fruits is a problem in the food industry as it decreases acceptance of food. Bitterness deters consumers from ingesting beneficial phytonutriments which would lower their risks of suffering from cardiovascular diseases, obesity, diabetes or cancer (Peyrot des Gachons *et al.* 2009). As bitterness is a problem in the food industry, a number of debittering methods and natural or

synthetic taste masking molecules are developed to diminish or eliminate the bitter taste (Drewnowski and Gomez-Carneros 2000; Ley 2008). Amongst these methods, the addition of sugar or sweeteners remains one of the simplest way to mask bitterness (Wilkie *et al.* 2013; Mennella *et al.* 2015) as sweetness at moderate to high intensities is generally suppressive of other basic tastes (Keast and Breslin 2002).

Most of these bitter compounds are aversive to insects, whether they are phytophagous (Schoonhoven *et al.* 1992; Schoonhoven and van Loon 2002; Chapman 2003), detritiphagous (Dethier 1976; Dethier and Bowdan 1992; French *et al.* 2015a and refs. therein), or blood feeders (Kessler *et al.* 2013; Kessler *et al.* 2014; Pontes *et al.* 2014). Numerous examples show that bitter substances inhibit sugar detection in insects. For example, in flies, alkaloids suppress sugar detection (Dethier and Bowdan 1989; 1992), and 1 mM quinine is enough to completely inhibit the peripheral detection of 35 mM fructose in *Drosophila* adults (Sellier *et al.* 2011). Such suppression is due to peripheral and central components. As in Vertebrates, the inhibitory effect of bitter chemicals depends upon satiety level, hungry insects accepting to feed on higher concentrations of bitter substances than satiated insects (Dethier 1976; Farhadian *et al.* 2012; Marella *et al.* 2012). This response is centrally regulated through conserved pathways between flies and mammals, *e.g.* neuropeptide and insulin-like signaling (Wu *et al.* 2005). In addition, local circuits fine tune the integration of taste information in the suboesophageal ganglion through GABA interneurons (Chu *et al.* 2014; Pool *et al.* 2014). At the periphery, the responses of sugar-sensitive neurons are inhibited by alkaloids (French *et al.* 2015b), an inhibition which might be mediated or facilitated by specific OBPs as shown by Jeong *et al.* (2013) with OBP49a.

Sweet suppression of bitter taste in insects is less documented. Simmonds *et al.* (1990) reported that the detection of azadirachtin is inhibited by sucrose in *Spodoptera littoralis* larvae, but that the detection of other alkaloids is not affected by increasing concentrations of sucrose. Inositol and sucrose suppress the detection of sinigrin in *Mamestra configurata* and *Trichoplusia ni* larvae (Lepidoptera) (Shields and Mitchell 1995). Inositol (but not sucrose) masks the taste of caffeine in *Manduca sexta* larvae (Glendinning *et al.* 2000). More recently, Cocco and Glendinning (2012) showed that sweet suppression occurs in *M. sexta* larvae, when sucrose, inositol, or glucose is mixed with caffeine or aristolochic acid. Furthermore, they found that binary mixtures of sugars are more effective in suppressing bitter taste than single substances, and that sugars differ considerably in their capacity to mask bitter taste.

Here, we examine whether sugars could alter the detection of bitter substances in *Drosophila*. We selected four aversive compounds which differ by their detergency, *e.g.* denatonium > berberine > umbelliferone > caffeine according to Weiss *et al.* (2011). These aversive compounds were mixed with 12 sugar molecules, which differ in their sweetness and nutritive power according to Hassett (1948). We first recorded if short term consumption of flies was differently affected by these bitter substances according to the identity and the concentration of the sugar molecules. Interestingly, caffeine was the most inhibitory over the whole panel of sugars. We then asked if sugars could alter the response of bitter-sensitive neurons, by recording the electrophysiological responses from two i-type labellar sensilla which host only two cells of opposite modality (Hiroi *et al.* 2004). This makes easier to distinguish the activities from bitter- and sugar-sensitive cells within extracellular recordings. We observed that almost all sugars significantly inhibited the activity of bitter-sensitive cells at 100 mM. However, only four sugars (maltotriose, sucrose, glycerol and maltose) elicited strong responses in the sugar-sensitive cells in the presence of bitter substances. Lastly, we evaluated if bitter-sensitive cells could be inhibited by sugar-sensitive cells, by comparing the responses of flies with ablated sugar-sensitive cells to that of normal flies. We recorded the gustatory responses of i9 sensilla to mixtures of caffeine and theophylline with sucrose, glycerol and a mixture of the two. We found that both control and mutant flies showed a reduced response of their bitter-sensitive cells with 10 mM sucrose or 10% glycerol, and that a mixture of the two sugars was more effective than single sugars. Our observations demonstrate that most sugars exert a

suppressive effect on the detection of bitter chemicals and that this inhibition is not dependent on the activity of sugar-sensitive cells.

Materials and Methods

Drosophila stock and transgenes. Flies were maintained on a standard axenic medium at 75% - 80% humidity, and 25°C ± 1°C on a 16:8 light-dark cycle. Only females were used for electrophysiological tests. Both males and females were used for the behavioral tests. The flies tested were 2-6 d old. White type flies *w¹¹¹⁸*, *UAS DTI*, and *Gr5a-Gal4* flies were from Kristin Scott (UC Berkeley, USA).

Chemicals. All molecules were from Sigma Aldrich purchased at the highest grade. Bitter molecules were used at the following concentrations: 10 mM caffeine (CAF), 0.1 mM berberine chloride (BER), 0.1 mM denatonium benzoate (DEN), 10 mM theophylline anhydrous (TPH) and 10 mM umbelliferone (UMB). These concentrations were determined following pilot experiments which suggested that these molecules would elicit between 20 and 30 spike/s from i8 and i9 sensilla. The sugar molecules were sucrose (SUC), D- trehalose dehydrate (TRE), Inositol (INO), D-galacturonic acid (GALA), D-melezitose hydrate (MEL), D-maltose hydrate (MALT), maltotriose hydrate (3MALT), D-fructose (FRU), D-sorbitol (SORB), D-glucose anhydrous (GLU), glycerol (GLY), D-arabinose (ARA). The sugar molecules were used a 1, 10 or 100 mM (or 0.1, 1, 10 % for glycerol). All solutions were stored at -20°C aliquots and kept at 4°C for no more than one week. For electrophysiology, the solutions were completed with 1 mM KCl or 30 mM tricholine citrate (TCC), which served as an electrolyte and contributed to inhibit the activity from the water cell (Wieczorek and Wolff 1989). For behavioral observations, the solutions were completed with erioglucine 125 mg/100ml in order to improve the detection of the liquid levels within capillaries.

Behavioral assay. Assays were performed according to Sellier *et al.* (2011). Briefly, freshly emerged flies (1-2 d old) were transferred to fresh food for one day and starved for 20-22 h with access to water. Then, flies were divided into groups of 20 flies and transferred into a rectangular transparent plastic boxes (95x76 x15 mm, Caubère, France). Each box contained 5 capillaries (5 µl, Hirschmann Laborgeräte, VWR) which were disposed on a microscope slide with double sided sticky tape. Each capillary was filled with a test solution with a blue dye (Figure 2). Flies were kept in the dark for 2h at 25°C (Sellier *et al.* 2011). A box without flies was used as a control to monitor evaporation. A picture of each box was taken on a flatbed scanner at the beginning and at the end of an experiment, and the length of the liquid within each capillary was measured using ImageJ (Abramoff *et al.* 2004).

Electrophysiological recordings. Electrophysiological recordings were performed as in French *et al.* (2015b). Briefly, 2-6 d old flies were collected and briefly anesthetized on ice. They were immobilized on putty (yellow Patafix, UHU) placed on a cylindrical magnet, with fine stripes of tape, in order to expose sensilla of the labellum. A drop of electrocardiogram gel was disposed between the abdomen and a silver wire connected to the ground. Individual taste sensilla on the proboscis were stimulated by covering their tip during 2 s with a capillary electrode containing a stimulus dissolved in 1 mM KCl or 30 mM TCC. Stimuli were separated by a time interval of 2-5 min in order to avoid adaptation and contamination from the previous stimulus. The sensilla recorded were selected and named according to Hiroi *et al.* (2002). The electrical signals were amplified with a taste probe (Marion-Poll and van der Pers 1996), further amplified and filtered (x500 – 1000; 10-2800 Hz: CyberAmp 320, Axon Instruments, USA), digitized at 16 bits, 10 kHz (DT9800, Data Translation, USA) and further stored and analyzed using a custom program, dbWave (Marion-Poll 1996).

Experiment 1. We asked if mixing these bitter molecules with sugars at different concentrations can reduce the consumption of flies and if specific mixture combinations were less deterrent than others. Groups of 20 flies were given a choice between 5 capillaries, 4 capillaries containing either of

the sugar molecules (at the same concentration: 1, 10 or 100 mM; 0.1%, 1%, 10% for glycerol) mixed with a bitter substance (denatonium, berberine: 0.1 mM; umbelliferone, caffeine: 10 mM). Sugars were tested in three combinations: (a) fructose, melezitose, galacturonic acid and maltotriose, (b) sorbitol, inositol, glucose and trehalose, and (c) sucrose, glycerol, arabinose and maltose. A fifth capillary contained the same bitter substance that was mixed with the sugars, 1, 10 or 100 mM. As for the MultiCAFE test, each box contained 4 capillaries filled with either of these sugars mixed with one bitter substance and one capillary filled with the bitter substance. The sugars within each box were presented at the same dilution 1, 10 or 100 mM. Ten repetitions of each combination were tested (3 groups of 4 sugars (a,b,c) x 3 concentrations).

Experiment 2. In order to evaluate if mixtures of these molecules were detected independently by separate taste cells, we recorded the electrophysiological responses of i8 and i9 sensilla to the same molecules that were tested behaviorally. Only one series of concentrations was tested on a single sensillum in the following sequence: (a) bitter, (b) bitter + 1 mM sugar, (c) bitter + 10 mM sugar, (d) bitter + 100 mM sugar, (e) bitter, (f) 100 mM sugar. According to the results of experiment 1, i8 sensilla were stimulated with denatonium, berberine and caffeine, while i9 sensilla were stimulated with umbelliferone and caffeine. Each combination of sugar (n= 12) x bitter (n=4) was tested 10 times.

Experiment 3. In order to evaluate if sugar-sensitive cells participate to the suppression of the bitter taste, we performed a series of electrophysiological observations on i9 sensilla of flies in which the sugar-sensitive cells were genetically ablated. To do so, we crossed two parental lines respectively carrying Gr5a-Gal4 and UAS-DTI (Wang *et al.* 2004). These flies (hereafter denoted Gr5a > DTi or mutant flies) and their parents were tested with 10 mM caffeine or 10 mM theophylline mixed with increasing concentrations of glycerol (0.1, 1, 10%) or sucrose (1, 10, 100 mM). Such flies were also tested for a combination of sugars, by stimulating them with caffeine or theophylline mixed with sucrose and glycerol. Ten repetitions of each situation were recorded.

Data analysis. Data from the multiCAFE tests were measured and corrected for evaporation by measuring the volume of liquid lost in boxes without flies but prepared along with the other boxes in the same way. The corrected consumption was subjected to a two-ways ANOVA analysis.

Electrophysiology data were measured as number of action potentials emitted during the first second of each recording. Putative action potentials were detected by adjusting a threshold over a filtered version of the signal (a 30 points running median) and by extracting 60 points each time the signal crossed the threshold. The waveforms were subsequently sorted by interactive procedures under dbWave (Marion-Poll 1996; Meunier *et al.* 2003). As i-type sensilla are housing 2 gustatory cells and one mechanosensory cell, spikes were sorted in 3 classes on the basis of their amplitude and spiking frequency. As each stimulus series included the best stimulus for each cell, we could check the validity and consistency of our sorting procedures over all recordings obtained from the same sensillum. Data from experiment 3 were first tested with a Friedman's test of paired values and then with an ANOVA test. Data from experiment 4 were tested with Friedman and Dunn's multiple comparisons tests of paired values. The statistical values were computed using GraphPad Prism 6 (Friedman and Dunn's multiple comparison tests) or Statistica 10 (ANOVA analysis).

Results

Experiment 1

Flies given access to sugars mixed with a bitter substance consumed different amounts of the solutions depending on the combination of the 3 factors bitter molecule x sugar molecule x sugar concentration. Among the four bitter substances, 10 mM caffeine was the most inhibitory, while 0.1 mM denatonium, 0.1 mM berberine and 10 mM umbelliferone yielded similar levels of consumption (Figure 1 A). Across all experiments, flies fed significantly more of the solutions with increasing

concentrations of sugars (Figure 1 B). Amongst sugars, maltotriose, glucose, sucrose were the most stimulatory, while sorbitol, galacturonic acid and arabinose were almost not consumed (Figure 1 C). Further comparisons are hampered by the fact that we used fixed combinations of sugars.

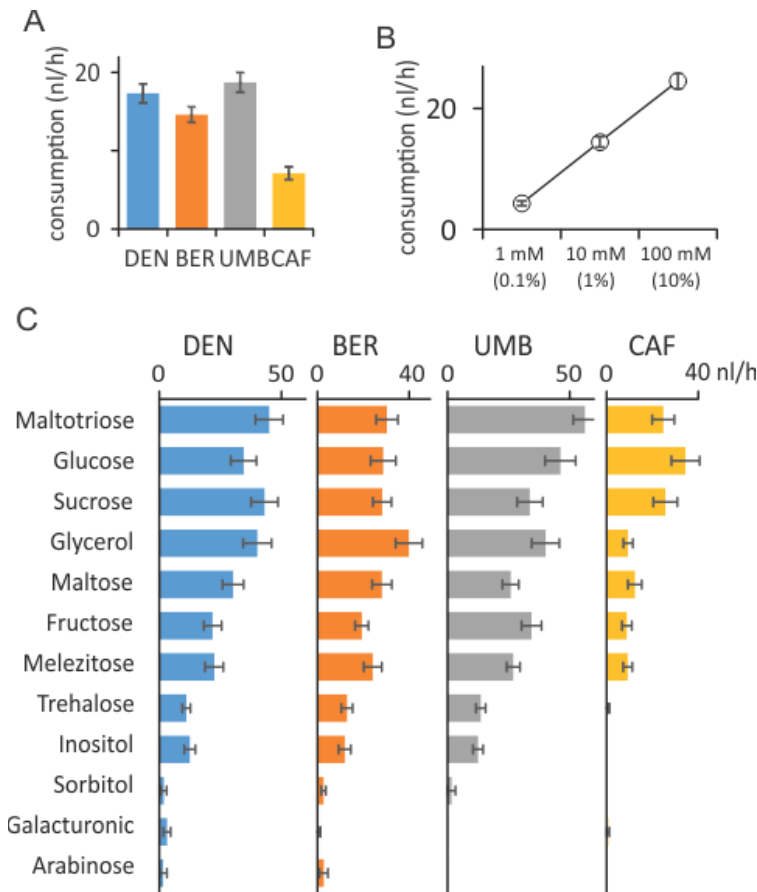


Figure 1: Influence of bitter molecules, sugar concentration and sugar molecules upon consumption.

Group of 20 flies were given access during 2 h to 6 capillary tubes containing either 0.1 mM denatonium (DEN), 0.1 mM berberine (BER), 10 mM umbelliferone (UMB) or 10 mM caffeine (CAF) mixed with 1 sugar (out of 12 tested) at 1 concentration (1, 10 or 100 mM). As each situation was reproduced 10 times, the entire set of data represents 10 x 12 sugars X 3 concentrations x 3 bitter substances. In this figure, the influence of the bitter substance, of the concentration of sugar and of the sugar are displayed separately by computing the respective averages over the entire set of data. (A) Influence of denatonium, berberine, umbelliferone and caffeine upon the consumption of sweet molecules (3 different concentrations 1, 10, 100mM for each of the 12 sugars tested or 1.0, 1, 10 % of glycerol). (B) Influence of the sugar concentration upon the consumption of bitter substances. (C) Influence of each sugar upon the consumption.

Experiment 2

We examined the responses from i8 and i9 sensilla to the same set of bitter and sugar molecules. These sensilla host only 2 neurons which allow us to separate the activities of the neurons within extracellular records obtained by capping the tip of one sensillum. The neural activities from i8 and i9 sensilla could exhibit two classes of action potentials. One class was active in response to bitter substances like caffeine, and showed large spikes (1-5 mV). The other class was activated by sugars like maltose and showed spikes of smaller amplitude (0.3-1 mV) (Figure 2). These amplitudes could drift with the firing activity of the neuron and were smaller with mixtures (Figure 2). These two cells are called thereafter as L cell (large spike cell) and S cell (small spikes cell).

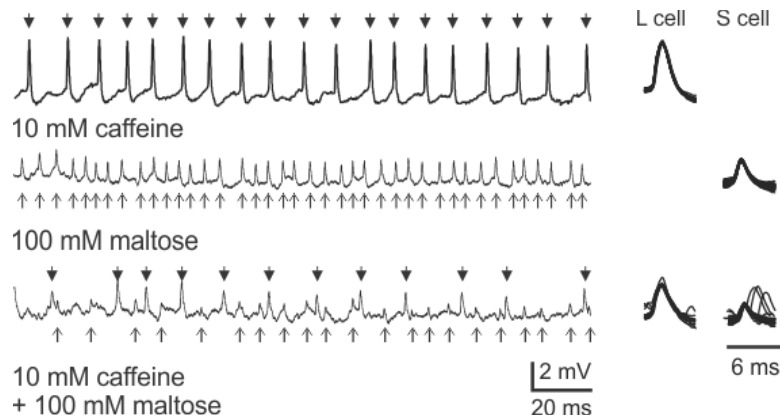


Figure 2: Sample responses to maltose and caffeine in *i9* sensilla which activate two different classes of spikes.

Sample recordings obtained from *i9* sensilla using a tip-recording electrode. The central panel displays a selected portion of 200 ms of a 2 s recording. The spikes detected are pointed by an arrow placed over or below the trace. The right panel shows a superposition of the spikes detected in the central panel – the left column regroups spikes classified as “large” and the right column, the spikes classified as “small”. The vertical scale of these curves is indicated by a bar below the central panel. (A) 10 mM caffeine elicit spikes from one cell with large spikes (L cell). (B) 100 mM maltose elicit small amplitude spikes (s cell). (C) When 10 mM caffeine is mixed with 100 mM maltose, two classes of spikes are found which correspond respectively to the activities of the s and L cells. The spikes are notably smaller however than in the two previous situations. On the right panel, we see that some small spikes are followed by the firing of a larger spike, confirming that two cells are active at the same time.

In the absence of sugar, L cells responded with about 20 spikes/s to 0.1 mM denatonium and berberine, and with 25 spikes/s to 10 mM caffeine in *i8* sensilla; in *i9* sensilla, the cells respond to 10 mM umbelliferone with 28 spikes/s and to 10 mM caffeine with 35 spikes/s. The responses of S cells to 100 mM sugars were between 0 and 100 spike/s depending on the molecule.

We computed average responses over the whole set of recordings ($n = 3847$), in order to evaluate the effect of the bitter treatment on the responses (Figure 3 A). The influence of each bitter substance on the responses of L- and S-cells was quite similar. We also computed the responses of L- and S-cells to increasing concentrations of sugar. We observed a reduction of the firing activity of the L cells in relation to the sugar concentration of about 25% (Figure 3 B). Inversely, in the presence of bitter molecules, the responses of S cells to 100 mM sugars was reduced down to 55 % (*i8*) and 61% (*i9*) of the responses without sugars.

We analyzed the reduction of firing activity of L cells, induced by individual sugars by comparing the responses of the mixtures to the responses to the bitter substance alone. Almost all sugars exhibited an inhibitory activity at 100 mM (Figure 3 C). For denatonium, the largest reductions were recorded with maltotriose and melezitose. With berberine, maltotriose, glycerol, sucrose and maltose were the most inhibitor. Umbelliferone was mostly inhibited by maltose and galacturonic acid. With caffeine, the most efficient molecules were maltose, glycerol melezitose and galacturonic acid.

We also compared the responses of S cells toward 100 mM sugar and the responses to the mixture. First, the firing activities elicited by the sugars alone differ greatly with the molecules. Inositol and sorbitol elicit very few spikes, while maltose, maltotriose, sucrose, glycerol and arabinose are quite stimulatory.

In summary, stimulating *i*-type sensilla with a binary mixture of a bitter compound and a sugar induces most of the time a reciprocal inhibition of the respective responses of L-cell and S-cells.

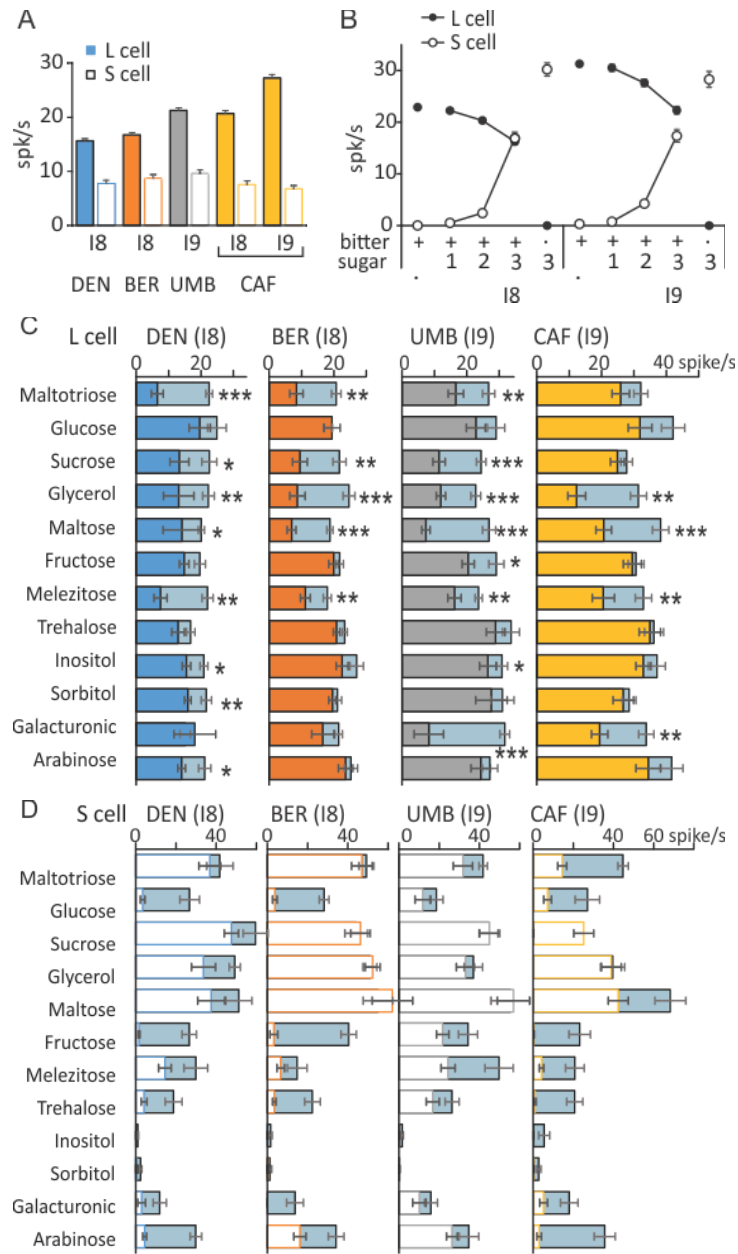


Figure 3: Responses of i8 and i9 sensilla to single bitter or sugar molecules.

We used the same combination of mixtures of bitter and sugar stimuli to stimulate individual taste sensilla of the proboscis (i8 and i9) which house only 2 cells. In the same way as in figure 31, the average effects of bitter molecules, of the concentration of sugar and of each sugar is displayed in separate figures. We measured the activities of sugar-sensitive and bitter-sensitive cells and display here the number of spikes recorded during the first second of the recordings. The number of measures is n=10 for each combination of stimuli (4 bitter x 12 sugars x 4 sugar concentrations 0, 1, 10, 100 mM) (A) Effects of each bitter molecule upon the responses of cells within i8 and i9 sensilla. Filled bars represent the responses of bitter-sensitive cells (large spikes). The amplitude of the responses are slightly different. Caffeine was tested both in i8 and i9 sensilla. Empty bars show the average responses of sugar-sensitive cells (small spikes). (B) Effect of sugar concentration upon the responses of the bitter (black dots) and sugar (empty dots) cells. (C) Influence of the sugar molecules upon the responses of the bitter-sensitive cells to mixtures of denatonium, berberine, umbelliferone and caffeine. Colored bars (blue, orange, grey and yellow) represent the average responses in the presence of sugar. The blue-grey bars display the average response of the bitter-sensitive cells to the

bitter substance alone. **(D)** Influence of the sugar molecules upon the responses of the sugar-sensitive cells in a mixture with denatoniu, berberine, umbelliferone or caffeine (empty bars) or alone (blue-grey bars). Each bar or point represents the average of the number of spikes observed during the first second of the recordings. The brackets show the SEM. MANOVA tests were used to check the significant difference between the control condition (no mixture) and the response to the mixture.

Experiment 3

As the previous results rely upon an experienced observer to sort the spikes, we confirmed that sugars can inhibit bitter detection by using a genetic construction to eliminate the S cell. This was obtained by crossing flies carrying a promotor sequence of a sugar receptor, *Gr5a*, to drive the expression of GAL4, together with flies carrying as a reporter gene, the diphtheria toxin *DTI*. We used both parents (*Gr5a-Gal4* and *UAS-DTI*) as control lines and compared their response to the F1 progeny (*Gr5a > DTI*).

As found in the previous experiment, i9 sensilla responded to 10 mM caffeine with large amplitude action potentials. When caffeine or theophylline were mixed with increasing concentrations of sucrose or glycerol, the response of L cells decreased in the mutant flies as well as in the parental lines (Figure 4 A). The L cells responded with increasing spiking activity to higher sugar concentrations in the parental lines but not in the mutant flies (Figure 4 B).

We further tested a mixture of the two sugars, mixing glycerol with sucrose. We observed a significant reduction of the responses to caffeine and theophylline in the mutant flies as well as in the parents (Figure 4 C). The S cell showed a parallel increase of firing activity in the parental lines but not in the mutant (Figure 4 D).

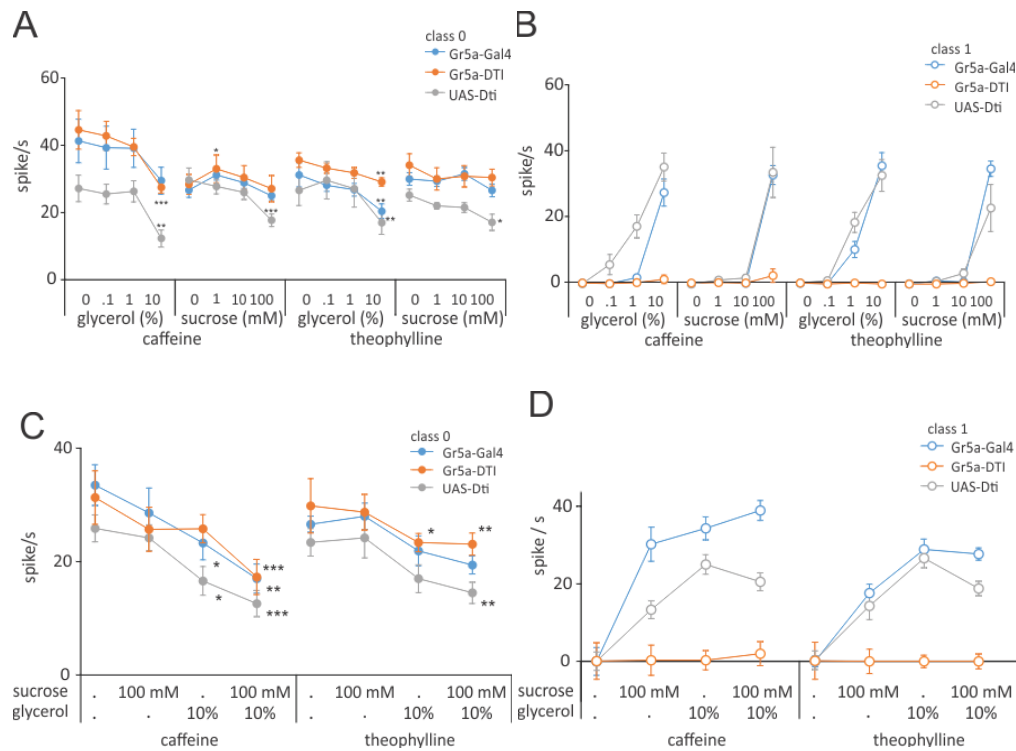


Figure 4: Sugar inhibition of bitter responses in flies with ablated S-cells

In these two experiments, we compared the responses of bitter sensitive cells in the presence or absence of a sugar-sensitive cell towards (a) caffeine or theophylline mixed with different concentrations of sucrose and glycerol, or to a combination of these two sugars at a single concentration to seek for an additive effect. Sugar-sensitive cells were ablated using a genetic construction, by expressing the diphtheria toxin (DTi) into sugar sensitive cells expressing *Gr5a*. In the mutant flies (denoted *Gr5a>DTi*), the sugar sensitive cells were ablated while they were functional in the parental lines (denoted *Gr5a-Gal4* and *UAS-DTi*). **(A)** Responses of bitter-sensitive cells (L cells) toward 10 mM caffeine or 10 mM theophylline mixed with glycerol (0, 0.1, 1, 10%) or sucrose (0, 1, 10, 100 mM). **(B)** Responses of sugar-sensitive cells to the same stimuli. **(C)** Responses of bitter-sensitive cells to caffeine or theophylline mixed with either 100 mM sucrose or 10% glycerol or to both sugars. **(D)** Responses of sugar-sensitive cells to the same stimuli.

Discussion

In this work, we evaluated if sugars could inhibit the response of bitter-sensitive cells in flies. We first checked if flies could overcome the aversive taste of 4 bitter substances when mixed with a panel of sugars. We observed that flies consume more of the bitter solutions with increasing concentrations of sugar. We then looked at the gustatory responses of taste sensilla on the proboscis, and observed that the responses to mixtures of bitter and sweet substances were in general lower than the responses to the isolated compounds. By separating the respective activities of the bitter and of the sugar sensitive cells, we showed that sugars and bitter molecules have reciprocal inhibitory effects. While the inhibition of the bitter sensitive responses were about 25 % at the maximal concentration tested, the responses to sugars were strongly diminished up to 60%. Furthermore, while it seems that almost all sugar tested can exert an inhibition, the inhibition of the four bitter substances was very different according to the sugar molecules. Lastly, we show here that a mixture of two sugars is more efficient than a sugar alone.

This is not the first time that such observations are reported in insects but nevertheless sweet inhibition has not received much attention in insects. The most recent observations were made on the gustatory sensilla of larvae of a Lepidoptera, *Manduca sexta* (Cocco and Glendinning 2012). In this work, the authors used a short term biting assay to monitor the behavioral responses towards tastants. The behavioral assay used here is running over 2 h, which opens the possibility that the results observed are due not only to inhibition occurring at the periphery, but also by post-ingestive evaluation. A useful complement to this study would be to test proboscis extension responses or to limit the observation period to a shorter time. This might be one reason why the behavioral responses to the range of sugars tested differs from the electrophysiological observations, as the two set of data concern very different time scales, 2 h for the behavior and 2 s for electrophysiology.

Within the sweet/bitter mixtures, each neuron showed a reduced activity. While bitter inhibition has been reported many times in insects and in *Drosophila* ((French *et al.* 2015a), sweet inhibition has never been observed in the flies. We took advantage of a specific feature of some taste sensilla of the proboscis that host only 2 gustatory neurons to analyze in more detail how sweet inhibition happens. Although this preparation is more favorable as regards spike separation, spike detection and sorting remains difficult in *Drosophila*. One of the reasons is that the amplitudes of the spikes fired from a given neuron vary with time and with the spiking frequency of the neuron (Fujishiro *et al.* 1984). Another reason is that the background noise of the recordings vary from fly to fly. Lastly, although we assumed that 2 neurons only are present in i8 and i9 sensilla, it is still possible that some variability occurs. In this experiment, we did not find much variability in the number of cells active. However, this may be specific to our laboratory strain since Weiss *et al.* (2011) reported that one of the sensilla tested here was quite variable in their hands, and that additional sensilla were present on the proboscis of their strain.

We confirmed that reciprocal inhibition occurred by ablating one of the taste cells within i9 sensilla and by showing that sugars do inhibit bitter detection in the absence of S cells. This result is important for two reasons. First, it confirms the observations we made, which were based on manual sorting of spikes from control insects. Second, it also means that this inhibition is not due to an interaction between gustatory cells of the same sensillum as suggested by Cocco and Glendinning (2012). While the demonstration made here is less comprehensive than previously done for bitter inhibition (French *et al.* 2015b), this suggests that lateral interactions within neurons of the same sensilla do not represent the main mechanism by which sweet or bitter inhibition occur. While bitter inhibition can be partly explained or facilitated by OBPs (Jeong *et al.* 2013), especially for bitter substances which are amphiphilic and poorly water soluble, such a mechanism does not seem likely for sugars which dissolve really well in water. One possibility is that bitter and sweet molecules could associate to the gustatory receptors and thus diminish their responsiveness.

Our observations demonstrate that peripheral interactions occur in the gustatory system of flies and that these inhibitory interactions are quite significant. Sweet inhibition of bitter taste makes sense for nutritious sugars as it could allow insects to balance the benefit from ingesting nutritive food with the risk of ingesting noxious chemicals. However, similar inhibitions are observed with non-nutritious sugars such as arabinose. Therefore, the adaptive value of such interactions is probably limited. Further observations with a larger panel of sugars and molecules would be required to find more efficient bitter-masking molecules. Such molecules would be quite useful in crop protection, especially in “attract and kill” strategies strategy (Mangan and Moreno 2007; Witzgall *et al.* 2010; Reisenman *et al.* 2016), where they could contribute to mask noxious molecules.

Acknowledgments

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Abstract

Insect kinins (leucokinins) are multifunctional peptides acting as neurohormones and neurotransmitters. In females of the mosquito vector *Aedes aegypti* (L.), aedeskinins are known to stimulate fluid secretion from the renal organs (Malpighian tubules) and hindgut contractions by activating a G protein-coupled kinin receptor designated "Aedae-KR." We used protease-resistant kinin analogs 1728, 1729, and 1460 to evaluate their effects on sucrose perception and feeding behavior. In no-choice feeding bioassays (capillary feeder and plate assays), the analog 1728, which contains α -amino isobutyric acid, inhibited females from feeding on sucrose. It further induced quick fly-away or walk-away behavior following contact with the tarsi and the mouthparts. Electrophysiological recordings from single long labellar sensilla of the proboscis demonstrated that mixing the analog 1728 at 1 mM with sucrose almost completely inhibited the detection of sucrose. Aedae-KR was immunolocalized in contact chemosensory neurons in prothoracic tarsi and in sensory neurons and accessory cells of long labellar sensilla in the distal labellum. Silencing Aedae-KR by RNAi significantly reduced gene expression and eliminated the feeding-aversion behavior resulting from contact with the analog 1728, thus directly implicating the Aedae-KR in the aversion response. To our knowledge, this is the first report that kinin analogs modulate sucrose perception in any insect. The aversion to feeding elicited by analog 1728 suggests that synthetic molecules targeting the mosquito Aedae-KR in the labellum and tarsi should be investigated for the potential to discover novel feeding deterrents of mosquito vectors.

Leucokinin mimetic elicits aversive behavior in mosquito *Aedes aegypti* (L.) and inhibits the sugar taste neuron

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Insect kinins (leucokinins) are multifunctional peptides acting as neurohormones and neurotransmitters. In females of the mosquito vector *Aedes aegypti* (L.), aedeskinins are known to stimulate fluid secretion from the renal organs (Malpighian tubules) and hindgut contractions by activating a G protein-coupled kinin receptor designated “*Aedae*-KR.” We used protease-resistant kinin analogs 1728, 1729, and 1460 to evaluate their effects on sucrose perception and feeding behavior. In no-choice feeding bioassays (capillary feeder and plate assays), the analog 1728, which contains α -amino isobutyric acid, inhibited females from feeding on sucrose. It further induced quick fly-away or walk-away behavior following contact with the tarsi and the mouthparts. Electrophysiological recordings from single long labellar sensilla of the proboscis demonstrated that mixing the analog 1728 at 1 mM with sucrose almost completely inhibited the detection of sucrose. *Aedae*-KR was immunolocalized in contact chemosensory neurons in prothoracic tarsi and in sensory neurons and accessory cells of long labellar sensilla in the distal labellum. Silencing *Aedae*-KR by RNAi significantly reduced gene expression and eliminated the feeding-aversion behavior resulting from contact with the analog 1728, thus directly implicating the *Aedae*-KR in the aversion response. To our knowledge, this is the first report that kinin analogs modulate sucrose perception in any insect. The aversion to feeding elicited by analog 1728 suggests that synthetic molecules targeting the mosquito *Aedae*-KR in the labellum and tarsi should be investigated for the potential to discover novel feeding deterrents of mosquito vectors.

neuropeptide GPCR | sucrose taste | sensory neuron | chemical target validation | feeding deterrent

Females of *Aedes aegypti* (L.) mosquitoes (Diptera: Culicidae) are anthropophilic, feeding preferentially on blood from a human host, but both sexes feed on sugar-rich nectar as a source of metabolic energy. The female requires a blood meal for egg production and during that meal can transmit mosquito-borne diseases including dengue, Zika, chikungunya, and yellow fever viruses (1). Sugar feeding begins shortly after adult emergence and continues throughout adulthood. Importantly, sugar feeding influences vectorial capacity by increasing daily survival (2, 3) and can positively affect female reproductive maturation by increasing juvenile hormone synthesis (3).

However, ingesting liquids causes osmotic stress, which insects compensate through diuresis. We targeted this essential mechanism by altering neuropeptides affecting diuresis. Neuropeptide diuretic hormones increase the secretion of primary urine by the Malpighian tubules and increase hindgut contractions, which aid in fluid excretion (4). In *A. aegypti*, three endogenous kinins (aedeskinin I–III) act as diuretic hormones on Malpighian tubule stellate cells (5, 6) by stimulating chloride transport and fluid secretion (6–8). We verified that the aedeskinins activate the

single *Aedes* kinin receptor (*Aedae*-KR), a G protein-coupled receptor (GPCR) that signals through intracellular calcium (9). We designed kinin analogs to be resistant to degrading peptidases and therefore exhibit sustained high potency (10, 11). One biostable kinin peptidomimetic containing aminoisobutyric acid, 1728, has potency similar to or higher than the aedeskinins on recombinant receptors (12). Such biostable kinin analogs have potential in the control of insect pests because they reduce feeding in lepidopteran larvae (10, 13) and increase aphid mortality (14, 15).

Here, we examined whether three biostable insect kinin analogs, 1728, 1729, and 1460 (Fig. S1), affect feeding in female mosquitoes and/or have a direct impact on the gustatory detection of sugars. First, we demonstrate through feeding assays that the kinin analog 1728 significantly reduces the time females spend in contact with a sucrose solution and displays potent antifeedant activity. In addition, we present the first evidence, to our knowledge, that the potent kinin peptidomimetic 1728 further triggers female mosquito aversive fly-away or walk-away behaviors upon labellar and tarsal contacts with a sucrose source, overriding sweet taste perception. Electrophysiological recordings from the long labellar sensillum revealed that externally applied kinin analogs inhibited the sucrose-evoked response within milliseconds. Second, the *Aedae*-KR was cloned and sequenced from sensory

Significance

Kinin receptors are known in insects to contribute to osmotic regulation and are expressed in the excretory system, in the Malpighian tubules (renal organs), and in hindgut. We discovered that in *Aedes aegypti* mosquitoes, which are important vectors of human disease, a kinin receptor is also expressed within taste hairs (sensilla) on the legs and mouthparts. A kinin analog engineered to be peptidase resistant activates this kinin receptor with high potency, inhibiting sucrose taste detection directly at the level of the taste organs and eliciting a fast and highly aversive response in females during feeding. This finding suggests that mosquito G protein-coupled receptors could be new targets for discovering compounds to deter mosquitoes and preventing them from feeding.

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The authors declare no conflict of interest.

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labellar sensilla on the proboscis (Fig. 3A and Fig. S5A). In this preparation, extracellularly recorded spikes show an amplitude between 0.5 and 2 mV, depending on the insect and on the firing rate of the cells. The responses to 300 mM sucrose mixed with the kinin analogs (10 μ M to 1 mM of 1728, 1729, or 1460) generally showed only one class of action potentials (Fig. 3B), suggesting that these analogs do not activate another taste modality (i.e., salty or bitter), at least in the sensilla tested.

When the analogs were applied at 1 mM, a significant ($*P < 0.05$) decrease in the firing rate—60, 45, and 30% for analogs 1728, 1729, and 1460, respectively—was observed (Fig. 3A) as compared with sucrose only. Analogs were not inhibitory when applied at 10 μ M, but the level of inhibition was intermediate for 1728 and 1729 applied at 100 μ M. Analog 1460 inhibited the sucrose response significantly only when applied at 1 mM and was less inhibitory than the other two at this concentration, with analog 1729 being intermediate in potency (Fig. 3A). The representative traces obtained in response to sucrose in the absence or presence of the three analogs clearly show analog 1728 is the most potent, followed by 1729 and 1460 in that order (Fig. 3B). Kinin analogs depress both the phasic and the tonic portions of the responses to sugar, and this inhibition is concentration dependent (Fig. 3C). Kinin analogs thus exert their inhibition immediately after the female contacts the stimulus solution. The three analogs do not differ in their temporal kinetics, keeping the same rank order of potency, suggesting that they act on the same target. It is clear that the initial response to sucrose is higher than the initial number of spikes per second for analog 1728 at 100 μ M (and 1 mM); this difference is not so obvious for analog 1460. The response is very rapid for analog 1728 at 1 mM and is less rapid for

the other two analogs (Fig. 3C, black traces). These electrophysiological results are in accordance with observations of feeding behavior (Figs. 1 and 2, Figs. S2 and S3, and Movie S1), which indicated that analog 1728 was the most potent molecule.

To determine if analog 1728 had other aversive effects in addition to the inhibition of sucrose perception, we performed no-choice assays in the presence and absence of sucrose (Fig. S6). The time spent in contact with Evans blue only (E) was shorter the time spent in contact with sucrose solution containing Evans blue (SE). Importantly, we showed that Evans blue does not alter the number of spikes per second of the sucrose neuron (Fig. S5C), suggesting that females perceive solution (E) to be similar to water. In addition, there was no difference in the median time females spent in contact with the (E) solution or analog 1728 with Evans blue in the absence of sucrose (1728E). The median time spent in contact did not differ between 1728E and 1728SE, indicating that sucrose is not perceived differentially by these two female groups. However, the median time females spent with 1728SE was shorter than that spent with solution (E) (Fig. S6). These results clearly indicate that analog 1728 at 1 mM interacts strongly with the sucrose perception circuitry.

***Aedae*-KR Full-Length cDNA Cloning from Female and Male Legs and Female Labellum.**

The aversive response to analog 1728 appeared to be specific and mediated mainly by labellar and tarsal contact (Fig. 1 and Movie S1). Therefore, to investigate *Aedae*-KR expression in these appendages, full-length cDNAs were cloned from the labellum and legs of females and from the legs of males (Fig. S7). The *Aedae*-KR predicted amino acid sequences obtained were similar to the receptor cloned from the Malpighian tubule (AAT95982) (9), and the region selected for antibody production (residues 328–345; NEKFKREFHKRYPFGRN) (Fig. S7) also was identical. Thus, transcript expression of *Aedae*-KR was confirmed in appendages, validating the use of the previously reported antibody to localize the receptor (5, 7).

Immunolocalization of *Aedae*-KR and Identification of *Aedae*-KR Sensilla.

To verify *Aedae*-KR protein expression, immunohistochemistry was performed in frozen sections of labellum (Fig. 4 and Figs. S8 and S9) and tarsi (Fig. 5 and Fig. S10). The labellum is shown in Fig. 4A and Figs. S8A and S9A. The receptor signal (red) was observed in dendrites of sensory neurons extending to the tip of the long labellar hairs (Fig. 4) and in accessory cells present at the base of long labellar sensilla (Fig. 4 and Figs. S8 and S9), but the signal was detected only in accessory cells of short papillae (Fig. 4 and Fig. S8). Receptor signal was not observed at the base of hairs in the labellar proximal segment (Fig. 4 and Figs. S8 and S9). No signal was observed in tissues incubated with antigen-preabsorbed antibodies (Figs. S8 and S9) or preimmune serum (Fig. S9).

The *Aedae*-KR was immunolocalized in prothoracic tarsal sensory neurons in the second and third tarsomeres (Fig. 5A). Both tarsomeres exhibited a number of immunoreactive neurons along their proximal–distal axis; these neurons have somas that are strongly labeled by the anti-*Aedae*-KR antibody (anti-KR) and are close to the cuticle (Fig. 5B). *Aedae*-KR sensory neurons in tarsi appear to extend their dendrites (Fig. 5B, III-2) into sensilla trichodea (Fig. 5B, III-3). Negative control tarsal tissues, incubated with either antigen preabsorbed antibodies (Fig. S10A–C) or preimmune serum (Fig. S10D–F) did not show any receptor signal, as expected.

Effects of *Aedae*-KR Gene Silencing on Feeding Behavior. Gene silencing was performed to confirm that the mosquitoes' aversive behavior to the 1728 analog was mediated by the kinin receptor function in peripheral organs (Figs. 4 and 5). Two days postinjection of dsRNA, *Aedae*-KR expression was significantly reduced by 52% compared with control dsGFP-injected mosquitoes (Fig. 6A). In no-choice feeding assays with kinin analogs, control dsGFP females demonstrated aversive behavior to kinin analog 1728 at 1 mM (Fig. 6B), similar to that observed in naive 1728-challenged females (Fig. 1). In contrast, KR-silenced females no longer displayed the aversive

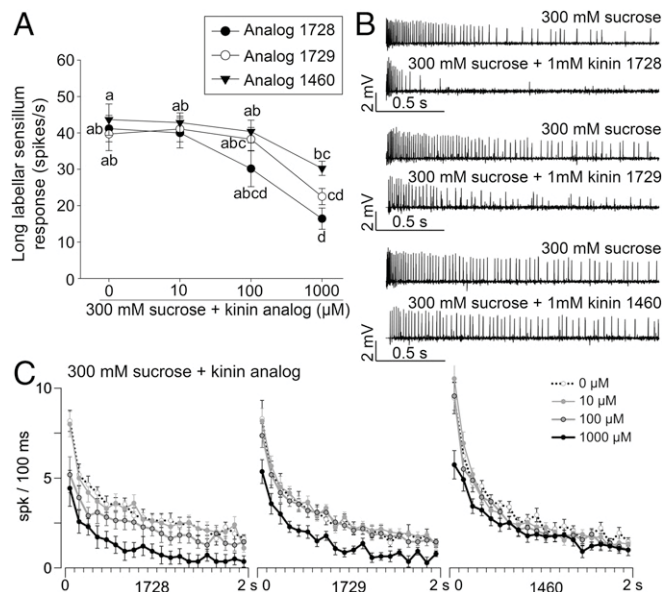


Fig. 3. Electrophysiological responses to kinin analogs recorded from long sensilla in the distal segment of the female labellum. (A) Long labellar sensilla were stimulated with kinin analogs 1728, 1729, and 1460 in 300 mM sucrose. For analogs 1728 and 1729 the maximal inhibition of the sucrose response was observed at 1 mM. Analog 1460 significantly inhibits the sucrose response only when applied at 1 mM, and it was less inhibitory than analogs 1728 and 1729 at the same concentration; analog 1729 was intermediate in potency. Data analysis (the number of spikes during the first second of 2-s recordings) was performed using the SAS command PROC generalized linear mixed model (GLMM) Tukey–Kramer test. Different letters indicate significant differences. Ten females were used to obtain each curve. (B) Consecutive responses to 300 mM sucrose (Upper Traces) and 1 mM of kinin analogs in 300 mM sucrose (Lower Traces) over 2 s. (C) Temporal dynamics of sweet neuron responses to kinin analogs at 1 mM in sucrose solution. In A and C each point represents the mean, and bars represent the SEM.

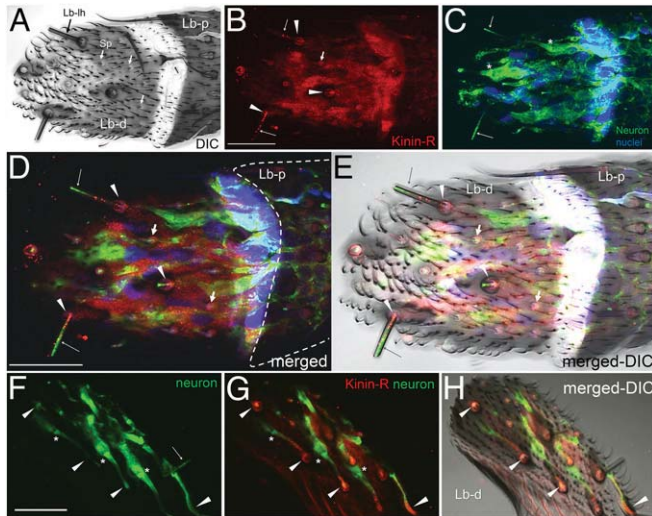


Fig. 4. Confocal analyses of the *Aedae*-KR immunolocalization in long labellar sensilla (Lb-lh, long hair) (black arrow) and short papillae (Sp) (white arrows) in the distal segment of the female labellum. Lb-d, labellar distal segment. (A) Diffused interference contrast (DIC) image. (B–H) The receptor signal (red) is present in dendrites of sensory neurons, indicated by long arrows in B, D, and E, and in accessory cells of both the long labellar sensilla, indicated by arrowheads in B, D, E, G, and H, and short papillae, indicated by short arrows in B, D, and E. Neurons appear green in C–H. Nuclei appear blue in C, D, and E. The root fiber bundles in the ciliary region of sensory neurons are marked by asterisks in C, F, and G. No receptor signal was observed in the labellum proximal segment (the area enclosed by a dashed white line in D). Images were acquired as Z-stacks (Z step: 0.41 μ m) using a 100 \times 1.4 oil immersion objective as follows: A and C, 20 sections; D and E, 11 sections; F, 19 sections. B, G, and H show a single optical section (depth, 4.51 μ m from the cuticle). (Scale bars, 20 μ m.) Two different tissues are shown in A–E and F–H, respectively.

behavior while probing and touching diets containing analog 1728 at a concentration of 1 mM (Fig. 6C). Consequently, the median time those silenced females spent in contact with the diet containing 1-mM analog 1728 (71 s) was similar to the time silenced females spent in contact with the sucrose solution (80 s) (Fig. 6C), suggesting that the KR is directly responsible for the aversion phenotype of the 1728 analog. Similarly, KR-silenced females exposed to analog 1729 at 1 mM did not display significant differences from those exposed to sucrose solution. Although no significant differences were found for the dsGFP females, a trend toward shorter time in contact with analog 1729 was observed for the dsGFP-silenced females (1729: 101.5 s vs. S: 129 s) (Fig. S11), as we had observed previously (Fig. 1).

Discussion

In this work, we clearly show, for the first time to our knowledge, that the gustatory detection of sugars is modulated by a neuropeptide directly at the level of peripheral sensory neurons. This finding suggests that kinin analogs interact with G protein-coupled receptors expressed within gustatory sensilla similar to the way that peripheral olfactory neurons are modulated by tachykinin (16, 17) and neuropeptide F (18–20). These peptides bind to GPCRs in olfactory receptor neurons (ORNs) or in circuits that modulate chemosensory signal-dependent feeding behaviors and food search (21). Here we provide, for the first time to our knowledge, evidence that neuropeptide GPCRs are expressed in taste peripheral neurons and that activating these GPCRs with kinin analogs changes the sensitivity of these neurons to sucrose.

Most mechanistic studies on insect kinins have been conducted with dipterans, *Drosophila melanogaster* and mosquitoes. *Drosophila* kinin is named “drosokinin” or “leucokinin” (*lk*) (22). Three pairs of *lk* subesophageal neurons (SELKs) receive projections from gustatory receptor neurons in the head (23, 24). Peripheral drosokinin

expression was found in sensory cells associated with tarsal sensilla (bristle sensilla) and in labellum by monitoring GFP expression driven by an *lk*-specific GAL4 line (23). Transgenic flies in which drosokinin release was blocked from the brain lateral horn (LHLK) and SELKs had altered olfactory and gustatory responses, respectively (24). Previous reports have questioned the significance of the *lk* system in taste perception in legs and labellum (25), based on the lack of GFP in mouthparts and legs in driver lines. The GFP-expressing cells shown by de Haro et al. (23) did not have the appearance of sensory neurons, and mutant flies did not display defects in the gustatory detection of sucrose (25). Therefore the significance of the kinin signaling system in peripheral taste sensory function in *Drosophila* is still controversial. There is no information about the peripheral functions of kinin neurotransmission in mosquitoes or about behavioral responses to altered kinin signaling in putative “leucokinin” or “leucokinin-responsive” sensory cells in labellum and legs.

Here, we exposed mosquitoes to sucrose solutions containing potent and biostable insect kinin analogs that are lethal to aphids and that are active on the recombinant *Aedae*-KR (12, 26). Floral nectar, which is fed upon by diurnal mosquitoes such as *A. aegypti*, contains ~55% sucrose, and both sexes prefer disaccharides, sucrose and trehalose, over monosaccharides (27). Because

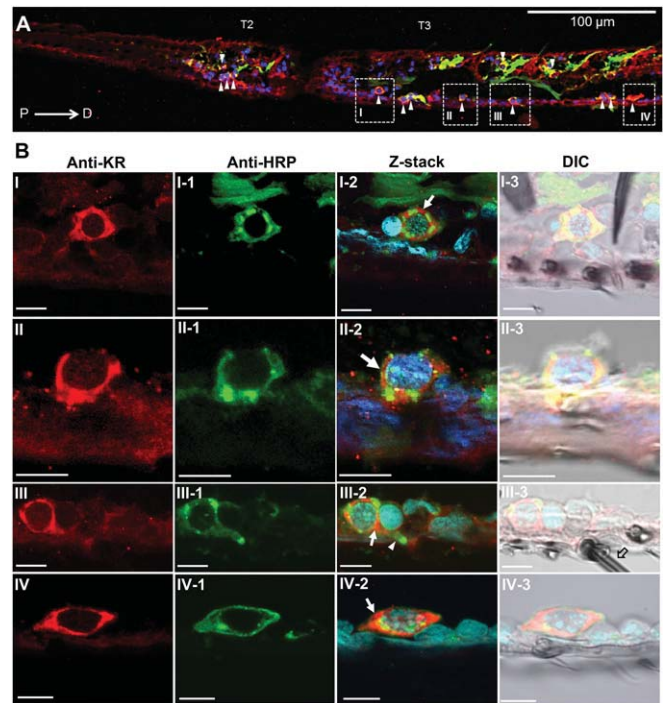


Fig. 5. Confocal analyses of the *Aedae*-KR immunolocalization in prothoracic tarsi. (A) Images of the kinin receptor signal (red, arrowheads) in sensory neurons (green) of the second (T2; four labeled neurons indicated by arrowheads) and third (T3; 10 labeled neurons indicated by arrowheads) tarsomeres. Tarsomeres are oriented from proximal (P) to distal (D) (left to right). The image is an x,y view of a 12.32- μ m Z-stack (eight Z-steps, each 1.54 μ m). (B) Images from the areas within the areas denoted by dashed boxes I–IV in A, taken using a 100 \times 1.4 oil immersion objective. The first two columns (B, I–IV and B, I-1–IV-1) show the red kinin receptor signal in the plasma membrane (B, I–IV) and cytoplasm of sensory neurons (green in B, I-1–IV-1). The merged images (Z-stack, B, I-2–IV-2) show receptor signal overlapping in neurons (red over green; arrows); nuclei appear blue. The DIC merged images in B, I-3–IV-3 show tarsal sensilla hairs and sockets very close to the receptor-labeled neurons. A receptor-expressing sensory neuron extends its dendrite (green; arrowhead in B, III-2) into a sensillum trichodea (open arrow in B, III-3). The first two columns (Anti-KR, Anti-HRP) show single sections (x,y); the last two columns show respective Z-stacks, as follows: B, I-2, 24 sections, Z-step: 0.25 μ m; B, II-2, 12 sections, Z-step: 0.41 μ m; B, III-2, 14 sections, Z-step: 0.41 μ m; and B, IV-2, 31 sections, Z-step: 0.25 μ m. (Scale bars, 5 μ m.)

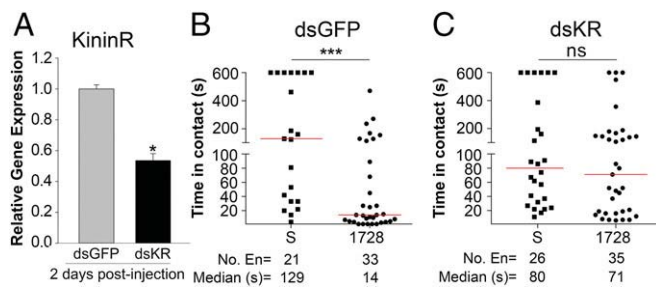


Fig. 6. Silencing of the *Aedae-KR* eliminates the aversive behavior elicited by contact with the 1728 kinin analog. (A) Relative gene expression was measured by quantitative RT-PCR (RT-qPCR) in control (dsGFP) and KR-silenced mosquitoes at 2 d postinjection. *Aedae-KR* expression was reduced significantly (~52%). Data are shown as the mean of three independent experiments \pm SEM. (B) Median time control dsGFP-injected mosquitoes spent in contact with sucrose solution or the kinin analog 1728 at a 1-mM concentration. (C) Median time dsKR-injected mosquitoes spent in contact with sucrose or kinin analog 1728 at a 1-mM concentration. Asterisks denote significant differences ($*P < 0.05$, $***P < 0.001$); ns, not significant. Statistical analyses were performed using GraphPad Prism with an unpaired t test to assess the efficiency of knockdown and a Mann-Whitney u test to evaluate the time in contact assays (B and C). Three independent RNAi experiments were performed.

most individuals accept a sucrose-only diet, we used a sucrose solution as a driver for providing the kinin analogs (27).

We discovered a previously unidentified function of the kinin-signaling system in mosquitoes in the rapid aversive response to the tasting/feeding of sucrose containing a kinin agonist, 1728, in 300 mM sucrose. The kinin analog 1728 contains pentapeptide kinin core residues (Phe- X^1 - X^2 -Trp-Gly-NH₂) identical to those of drosokinin (22), further supporting the role of insect kinins in chemosensory responses for taste perception of sugars, similar to that reported for trehalose in adult *Drosophila* (24). Before feeding, females normally touched diets with their labellum and tarsi but quickly avoided the agonist 1728 at 1 mM by flying, jumping, or walking away (Fig. 1 and Movie S1). Results from CAFE assays corroborated the observations that females significantly rejected this diet and also ate less diet with analog 1728 at 600 μ M (Fig. 2). This aversion also was reflected in plate assays showing that at these concentrations females retained less Evans blue (Fig. S3 A and B) and deposited fewer urine drops (Fig. S4). Similar results were obtained for analog 1729 at 1 mM in CAFE assays, but in plate assays the differences among analog concentrations in remaining Evans blue (Fig. S3 A and Inset II) and in the number of urine drops deposited (Fig. S4 B and Inset II) were not detected after 5 h. We then investigated if the aversive response to 1728 was correlated to the expression of the *Aedae-KR* in chemosensory appendages. The *Aedae-KR* immunostaining observed in the distal labellum (Fig. 4 and Figs. S8 and S9) coincides with dendrites of sensory neurons in the long labellar sensilla. These *A. aegypti* labellar hairs, from which recordings were also obtained (Fig. 3A and Figs. S5A, S8A, and S9A), are identical to the long labellar sensilla described in *A. aegypti* (28, 29) and are similar to the long labellar sensilla (trichoid) of *Anopheles gambiae* (30) and *Culiseta inornata* (31, 32). Accessory cells surrounding sensory neurons immunostained for the *Aedae-KR* could be the trichogen or tormogen (30, 33), because these cells are believed to secrete the dendrite bathing fluid (34, 35). The *Aedae-KR* staining at the sensilla base is reminiscent of accessory cells associated with sensory neurons in the proboscis of *Anopheles stephensi*, a mosquito vector of malaria (36).

We show that the *Aedae-KR* is expressed in sensory neurons associated with sensilla (Fig. 3 B, III-3) in the tarsi, where it was associated with tarsal sensilla trichodea (Fig. 3 B, III-2 and III-3). However, we cannot exclude the possibility that the receptor is present in neurons housed by other types of sensilla (37, 38). The *Aedae-KR* transcript expression and receptor localization in tarsi and in the distal segment of the labellum strongly linked the observation of the shorter time females spent in contact with

diet containing analog 1728 in feeding assays to the specific action of the kinin analog on the *Aedae-KR*. We performed single-sensilla recordings of long labellar sensilla in which the receptor was immunolocalized (Fig. 3A). Because *A. aegypti* prefer sucrose solutions of 100 mM or higher, a 300-mM sucrose solution was chosen for assays (27). Electrophysiological recordings on the long labellar sensillum of female *A. gambiae* showed that responses of the sucrose receptor cell reached a plateau at sucrose concentrations of 25–292 mM (10% sucrose) (30). We found that kinin analogs of diverse chemical structure inhibited the firing of neurons in response to sucrose in the female labellum (Fig. 3). The number of spikes per second decreased significantly in response to all three analogs at 1-mM concentrations, although 1728 appears to be the most potent (Fig. 3B). These results are consistent with the observation of reduced feeding of sucrose plus analog 1728 (Fig. 2). Moreover, using RNAi-mediated gene knockdown, we verified the fundamental role of *Aedae-KR* in mediating the aversive behavior resulting from contact with the 1728 analog. Females with silenced *Aedae-KR* no longer display aversive behavior in response to the 1728 analog at 1 mM (Fig. 6). This result strongly supports our conclusion that *Aedae-KR* expressed in sensory peripheral organs plays an important role in the control of feeding behavior. The kinin analogs tested have been characterized extensively in vitro and in vivo for their activity on insect kinin receptors. Two types of kinin analog structures were tested, the first containing α -amino isobutyric acid (Aib) (analog 1728 and 1729) and the second containing β^3 Pro (analog 1460) (Fig. S1). All activate recombinant mosquito and tick kinin receptors in CHO-K1 cells, with 1728 being the most potent on the *Aedae-KR* (EC₅₀, 76 nM), followed by 1460 (EC₅₀, 367 nM) and 1729 (EC₅₀, 625 nM) (12, 26). Analog 1728 and 1460 (12, 26) also have potent diuretic activity in the Malpighian tubules of *A. aegypti* in vitro (8). Although both the Aib-containing analogs 1728 and 1729 elicit hindgut contractions in *Rhodnius*; 1728 is more potent (39). Structural modifications of insect kinins, such as the incorporation of Aib and β -amino acids with an additional methylene group (-CH₂-), render these peptides biostable, because they are protease resistant (8, 10–12, 26). Our hypothesis is that the potent analog 1728 enters the labellar and tarsal sensilla (30) and diffuses through the aqueous sensillum lymph to activate the *Aedae-KR* expressed in sucrose taste neurons, thereby decreasing sucrose taste perception. We are not certain why analog 1728 elicits fast walk-, fly-, or jump-away behaviors (Movie S1) that are not observed for either analog 1729 or 1460, which also inhibit the sucrose response, although with less potency (Fig. 3C). It is possible that analog 1728 may stabilize the *Aedae-KR* in a specific conformation by homologous functional selectivity (biased agonism) (8). It is known that aedeskinins hyperpolarize the basolateral membrane voltage by increasing the chloride conductance of Malpighian tubule cells in *A. aegypti* (8, 40). A simple speculative explanation is that, acting via the *Aedae-KR*, analog 1728 changes the chloride concentration in the sensillum lymph, thus affecting the chloride channels involved in the repolarization of gustatory neurons and making the sucrose receptor cells insensitive (41). There is a lack of knowledge regarding chemosensory reception in mosquito leg sensilla. Our finding of the *Aedae-KR* in sensilla trichodea is supported by earlier studies showing that tarsal sensilla trichodea are involved in mosquito gustatory behaviors associated with sugar (42). In *Drosophila* the mapping of taste sensilla in tarsomeres revealed specific sensilla in tarsomeres 5–2 that detect sugars (43). Such a detailed map does not exist for any mosquito species. Our work contributes to the understanding of taste in mosquitoes by providing a receptor marker for a subpopulation of sensory cells in tarsi that can be pharmacologically manipulated and now can be explored further.

Our results pertaining to *Aedae-KR* peripheral function may extend the current knowledge about the modulation of ORNs by GPCRs in olfactory systems (discussed above) to those of chemosensory neural networks in taste organs such as labellum and legs, for which less information is available. Our pharmacological manipulation of the *Aedae-KR* by an externally applied

synthetic molecule provides proof of principle for the search of environmental deterrents (or repellents) modulating GPCRs in peripheral sensory neurons. Further, it demonstrates that these analogs are valuable tools for investigating how peripheral chemosensory systems define insect behavior. In mosquitoes the leucokinin system in peripheral organs appears to be involved in a behavioral avoidance mechanism in the context of sucrose feeding, in addition to its known role in the hormonal control of water and ion homeostasis. This avoidance mechanism could be present in other pests and might be exploited for their control.

Materials and Methods

Mosquito rearing was as described in ref. 5. Details of mosquito rearing, kinin analogs, no-choice feeding assays with kinin analogs, frozen-section immunohistochemistry of *Aedae*-KR in labellum and prothoracic tarsi, electrophysiological recordings on labellar long hair sensilla, the effects of gene

silencing on feeding behavior, dsRNA synthesis and the efficiency of knock-down, and statistical analyses are provided in *SI Materials and Methods*. The structures and synthesis of analogs are shown in Fig. S1. Primers are listed in Table S1.

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Supporting Information

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SI Materials and Methods

Mosquito Rearing. Larvae of *A. aegypti* (Diptera: Culicidae), Rockefeller strain, were reared at 26.5 °C under a 16-h light:8-h dark photoperiod in plastic trays (30 × 20 × 9 cm) with 2 L water. To obtain mosquitoes of consistent size, each pan contained ~200 larvae. Larvae were fed with Purina One Natural Blends (Nestle Purina PetCare). Pupae were kept in water in cups, which were placed in cages for adult emergence. Adults were fed using cotton wicks soaked in a 10% sugar solution (5).

Synthesis of Kinin Analogs. Insect kinin analogs containing Aib (1728 and 1729) or β-amino acid (1460) were synthesized as previously described (Fig. S1) (12, 26). Kinin analogs were solubilized in 100 μL of 80% acetonitrile containing 0.01% trifluoroacetic acid and were vacuum-dried and kept at –20 °C until use.

No-Choice Feeding Assays with Kinin Analogs. To determine whether agonist kinin analogs 1728 and 1729 have any adverse effect on mosquito females, these analogs were provided in a sucrose solution containing Evans blue (12, 26). Non-blood-fed (NBF) 3- to 5-d-old females were used for all feeding assays.

CAFE assays. Female mosquitoes were starved for 48 h but were provided access to water via a cotton wick inserted in a 25-mL flask. They were transferred by groups of five into fly glass vials. The vials were closed with a shortened plug, into which we inserted a single 5-μL calibrated capillary (32 mm in length) (VWR International) filled with the test solution. Each test solution contained 300 mM sucrose, 0.06% Evans blue, and kinin 1728 or 1729 at 0, 0.1, 0.3, 0.6, or 1 mM. The pH of the 1-mM solution of analog 1728 was <7 and ≥6.5. One tube was prepared with a capillary containing only sugar and Evans blue, without mosquitoes, to evaluate evaporation. Tubes were kept in a Plexiglas chamber at ~80% humidity and 26.7 °C. A picture of the group of tubes was taken at the beginning and at the end of the experiment. The volume of liquid in each capillary was measured using a ruler under ImageJ (44). The volume of liquid that disappeared during the observation period was converted into microliters per insect per hour. To evaluate better the effect of the analogs on actual feeding, we selected assays in which the volume of sugar solution that disappeared in the presence of mosquitoes was at least 1.5 times the volume of solution that disappeared in their absence (e.g., because of evaporation). For analog 1728 the results were analyzed by a one-way ANOVA (after checking normality with a Shapiro–Wilk test) followed by Dunnett’s multiple comparisons to the amount of liquid consumed in the sucrose-only treatment (45). For analog 1729 the results were analyzed by nonparametric Kruskal–Wallis ANOVA followed by Dunn’s multiple comparison test.

Plate assays. Analogs were solubilized in 50 μL of 10% sucrose solution containing 0.1% Evans blue (Alfa Aesar), pH 7.0 (hereafter referred to as “sucrose solution”). These assays were performed to evaluate the independent effect of each kinin analog. Diets were prepared by mixing 10% sucrose with either analog 1728 or 1729 (at 1 mM, 600 μM, 300 μM, 100 μM, 10 μM, or 1 μM). Twenty females were introduced into Petri dishes (100 × 15 mm) containing a single drop (50 μL) of one of the analogs at each concentration and one Petri dish containing only sucrose solution as control plate, making a total of seven plates per analog replicate. Three independent series were performed for each analog. Females’ behavior in these feeding assays toward the kinin analogs 1728 and 1729 at 1 mM and 600 μM and toward the sucrose solution was videotaped for 1 h, and the time that elapsed

from the onset of touching the solution [with the leg(s), the proboscis, or both] to flying or walking away from it was recorded. Data for the time spent in contact with the diets in plates containing analogs at 1 mM and 600 μM and sucrose solution were compared. At the end point (5 h) of these experiments, the total number of excreted drops (observed as blue deposits on the Petri plates) on the treatment plates of analogs 1728 and 1729 at 1 mM and 600 μM and the control sucrose solution was counted under a dissecting microscope (Fig. S4).

Additionally, for each plate, the remaining amount of ingested Evans blue in the 20 females was determined by their homogenization as a pool in 1 mL *N,N*-dimethyl formamide and incubation at 50 °C for 18 h (46). The samples’ OD at 620 nm was measured with a VersaMAX tunable microplate reader (Molecular Devices) and compared with those of an Evans blue standard curve (0.001–0.5 mg/mL).

Electrophysiological Recordings on Labellar Long Hair Sensilla. Females used for electrophysiology were a kind gift from Louis Lambrechts (Institut Pasteur, Paris). For electrophysiological experiments, 3- to 5-d-old females were anesthetized by being placed on ice for 1–2 min and then were immobilized sideways on putty (UHU patafix) using fine strips of tape placed over the body and the mouthparts to expose the 30- to 35-μm-long sensilla present at the tip of the proboscis, as in *A. gambiae* (Fig. S5A) (30). Recordings were obtained from dorsal and ventral long labellar hairs nos. 3–9, as mapped by Hill and Smith (28). The insect was grounded using a drop of electrocardiogram gel placed on the abdomen and was connected to a grounded silver wire (0.15 mm in diameter) (Fig. S5A). To stimulate individual taste sensilla, each sensillum was covered in turn by a capillary electrode (tip diameter 10 μm) (Fig. S5A) filled with the chemical to be tested and 30 mM tricholine citrate (TCC) (Sigma-Aldrich) in distilled water. TCC acts as an electrolyte and inhibits the activity of the water cells (47). We observed no significant response to this control solution (Fig. S5B). Because pilot experiments showed that Evans blue was inactive (Fig. S5C), we tested kinin analogs alone. The stimulus electrode was brought into contact with the sensillum for 2 s under visual control (Leica MZ12) with a micromanipulator (NMN-25; Narishige). The capillary touching the tip of a taste sensillum established an electrical contact. The signal was recorded through a nonblocking taste preamplifier (Tasteprobe DTP-02; Syntech) (48) connected to an amplifier (CyberAmp 320; Axon Instruments), where the signal was amplified further (100–1,000×) and filtered (eighth order Bessel filter, 10–2,800 Hz), digitized (16 bits, 10 kHz; A/D card DT9803; Data Translation), and stored on a disk for further analysis using a custom program (dbWave) (49). The number of action potentials in each recording was detected using a variable threshold adjusted over the digitally filtered recording (using a running median computed over 30 points on each side) and was exported to Excel for further analysis either as the number of spikes per second or by 100-ms bins.

mRNA Isolation and Cloning of *Aedae*-KR from Labellum and Legs. Labella were dissected from 3- to 5-d-old NBF females (*n* = 210). After the tissues were ground in liquid nitrogen, total RNA was isolated following the protocol of the NucleoSpin RNA manufacturer (Macherey–Nagel). Total RNA (0.1 μg) was used to synthesize 3’ RACE cDNA with the SMARTer RACE cDNA amplification kit (Clontech Laboratories). To obtain the full-length ORF cDNA of *Aedae*-KR from labellum, a PCR

amplification was performed using the RACE cDNA with the primers *Aedae*kinin5'UTR0 and *Aedae*kinin3'UTR1 (see Table S1 for the primers used in this section). The PCR program was as follows: 94 °C for 5 min; 40 cycles of 94 °C for 30 s; 59 °C for 1 min; 72 °C for 2 min; and a final extension at 72 °C for 5 min. The three pairs of legs from 3- to 5-d-old female ($n = 50$) and male ($n = 50$) mosquitoes were dissected, and mRNA was isolated following the protocol given in the Dynabeads mRNA Direct Kit (Invitrogen). Before the addition of lysate buffer (250 μ L) the legs were ground in liquid nitrogen. After mRNA elution, single-strand cDNA was synthesized using SuperScript III reverse transcriptase (200 U/ μ L) (Invitrogen). The cloning strategy to obtain the full-length cDNA from legs involved obtaining three PCR products. All PCR products were cloned in a TOPO TA cloning kit (Invitrogen) and were sequenced at the Gene Technologies Laboratory of Texas A&M University. First, the ORF of *Aedae*-KR after the seventh transmembrane region (TM#7) was amplified from these cDNA templates with *Aedae*kininF2 and *Aedae*kininR2 primers designed within the predicted ORF. PCR amplifications were performed with 94 °C for 5 min; 40 cycles of 94 °C for 30 s; 59 °C for 1 min; 72 °C for 2 min; and a final extension at 72 °C for 5 min. A product of the expected size (393 bp) was obtained. In addition, RACE cDNA was synthesized from mRNA isolated from female legs using the SMARTer RACE cDNA amplification kit (Clontech Laboratories). To obtain the 3' UTR and 5' UTR sequences of *Aedae*-KR, a first amplification was performed using the RACE cDNAs with universal primer mix (adaptor primer) and *Aedae*kininF2 and *Aedae*kininR2 primers for the 3' and 5' UTRs, respectively. Amplified PCR products were diluted (1:25) and used for a nested amplification with universal nest primer mix and *Aedae*kininF3 for the 3' UTR (the product obtained was 917 bp) and *Aedae*kininR3 for the 5' UTR (the product obtained was 1,861 bp). PCR conditions for both amplifications were 94 °C for 5 min; 40 cycles of 94 °C for 30 s; 61 °C for 1 min; and 72 °C for 2 min, with a final extension at 72 °C for 5 min. To obtain the ORF cDNA of *Aedae*-KR from legs in a single PCR product, either RACE cDNA from female legs or regular cDNA from male legs was amplified with the primers *Aedae*kinin5'UTR and *Aedae*kinin3'UTR0. PCR conditions were 94 °C for 5 min; 40 cycles of 94 °C for 30 s; 59 °C for 1 min; and 72 °C for 2 min, with a final extension at 72 °C for 5 min. Diluted PCR products (1:25) were used for a nested amplification with primers *Aedae*kinin5'UTR0 and *Aedae*kinin3'UTR1. PCR conditions were 94 °C for 5 min; 40 cycles of 94 °C for 30 s; 54 °C for 1 min; and 72 °C for 2 min, with a final extension at 72 °C for 5 min. The ORF was 1,789 bp.

Frozen-Section Immunohistochemistry of *Aedae*-KR in Labellum and Prothoracic Tarsi. Preparation of the anti-*Aedae*-KR antibody (anti-KR-Ct328-345, NEKFKREFHKRYPF RGRN) (5) and immunostaining procedures were performed as described in refs. 50 and 51, with modifications. Labellum and prothoracic tarsi were removed from 3- to 5-d-old NBF females ($n = 230$) and were fixed in 2% paraformaldehyde in PBS plus 0.1% Triton X-100 (PBST) for 1 h at 4 °C. After being washed in PBS at room temperature, the tissues were infiltrated with 12% sucrose in PBST for 8 h and for an additional 8 h with 25% sucrose in PBST (both steps at 4 °C). They then were embedded in Tissue-Tek optimum cutting temperature (O.C.T.) compound (Ted Pella) at room temperature for 10 min and were placed immediately inside the cryostat at -20 °C for at least 5 min. Frozen sections (14 μ m) were cut at -20 °C with a Leica Cryostat (Leica Microsystems) and were collected with a small brush on ColorFrost Plus microscope slides (Fisher Scientific). The sections on slides were dried at 37 °C for 24 h and were fixed by immersion in 4% paraformaldehyde in PBST for 30 min. After washing for 3 \times 5 min in PBST, the sections were incubated with blocking solution (PBST, 5% normal goat serum) for 1 h at room temperature. The sections were incubated with either anti-KR-Ct₃₂₈₋₃₄₅

(1:10 in blocking solution, 115 μ g IgG/mL) or anti-KR-Ct₃₂₈₋₃₄₅ preabsorbed antibody or preimmune serum (1:2,000) at 4 °C, overnight (7). The preabsorbed antibody was prepared in blocking solution with 500 μ g kinin C-terminal peptide (amino acid residues 328-345 in the receptor sequence; see above) per 40 μ g antibody (~1,000 molar excess). Neurons were labeled with Alexa Fluor 488-AffiniPure goat anti-HRP antibody (3 μ g IgG/mL; 1:500 in blocking solution) (Jackson ImmunoResearch) at 4 °C, overnight (in coinubation with primary antibody) (52). After washing for 4 \times 20 min in PBST, the sections were incubated with biotinylated goat anti-rabbit IgG (8.5 μ g/mL; 1:200) (Jackson ImmunoResearch) in blocking solution for 2 h at room temperature. Sections were washed 4 \times 20 min in PBST, incubated with Alexa Fluor 546 and streptavidin (10 μ g IgG/mL; 1:200) (Invitrogen) in blocking solution at room temperature for 1 h, and then washed 6 \times 30 min in PBST. Tissues were mounted with Vectashield Mounting Medium with DAPI (Vector), and images were obtained with either a Carl Zeiss Axio Imager A1 microscope (Carl Zeiss MicroImaging) or an Olympus FV1000 confocal microscope (Olympus America Inc.) at the Microscopy and Imaging Center at Texas A&M University.

Effects of Gene Silencing on Feeding Behavior.

dsRNA synthesis and gene silencing. The N terminus of *Aedae*-KR sequenced from female's legs was chosen as the target region of dsRNA. Specific primers flanked with the T7 promoter sequence were designed to amplify T7 DNA templates of *Aedae*-KR and GFP (Table S1). The MEGAscript RNAi kit (Life Technologies) was used for syntheses of dsRNA following the manufacturer's instructions. The dsRNA product was precipitated with ammonium acetate-ethanol and resolubilized in nuclease-free water to 5 μ g/ μ L. One-day-old NBF females anesthetized on ice were injected in the thorax with ~800 ng of dsRNA.

RNAi evaluation by RT-qPCR. Females ($n = 20$) at 2 d postinjection with dsRNA were homogenized in TRIzol reagent (Life Technologies), according to the manufacturer's protocol, to isolate total RNA. The isolated RNA was purified further with the RNA Clean & Concentrator-5 kit (Zymo Research) and was quantified using a NanoDrop spectrophotometer. cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) using 1 μ g total RNA. Expression of *Aedae*-KR was evaluated using either gene-specific primers or 18S rRNA primers (Table S1) (7). RT-qPCR was performed using the QuantStudio 3 Real-Time PCR System (Thermo Scientific) with PowerUp SYBR Green Master Mix (Thermo Scientific). A comparative cycle threshold ($2^{-\Delta\Delta C_t}$) method was used to assess relative transcript level (53).

RNAi evaluation by no-choice plate-feeding assay. Five days postinjection, dsGFP- or dsKR-injected female mosquitoes ($n = 20$ per treatment) were transferred into a Petri dish (100 \times 15 mm) after overnight starvation and were challenged with sucrose or a sucrose solution containing one of the kinin analogs at 1 mM as described above. The females' feeding behaviors were videorecorded during the first hour to evaluate the time spent in contact with the diet (from the onset of touching the diet to leaving it) at 27 °C and 80% relative humidity.

Statistical Analyses. Feeding bioassays were performed in three independent replicates. The time spent in contact with kinin analogs 1728 or 1729 at 1 mM and at 600 μ M or sucrose solution was analyzed by a nonparametric one-way ANOVA (Fig. 1) with GraphPad Prism 5.0 (GraphPad, Inc.). The treatment effect on the volume of Evans blue remaining per female was analyzed with the IBM SPSS Statistics 21 (SPSS, Inc.). Initially all data were analyzed by the univariate general linear model (GLM) considering analogs (1728 and 1729) and concentrations of analogs (1-1,000 μ M) as independent variables (factors). Subsequently we analyzed these data by one-way ANOVAs, followed by Tukey's honestly significant difference (HSD) test, as follows. First, for treatment effects, the two analogs 1728 and 1729 and sucrose solution treatments were compared when analogs were given at

the same concentration (independent ANOVAs from Fig. S3 *A–F*). Second, data for different concentrations of the same treatment (within-treatment concentration comparisons for analogs 1728 and 1729 and to sucrose) were compared by running two independent ANOVAs (Fig. S3, *Insets I and II*). Treatment effect on the number of deposited urine drops after 5 h (Fig. S4) was analyzed by a nonparametric one-way

ANOVA followed by the Kruskal–Wallis ranks test (GraphPad Prism 5.0). For analyses of electrophysiological recordings from long labellar sensilla (Fig. 3*A*), repeated-measures analysis of data (the number of spikes during the first second of 2-s recordings) was performed using the PROC GLMM Tukey–Kramer test. Data were analyzed by SAS 9.4 software (SAS Institute Inc.).

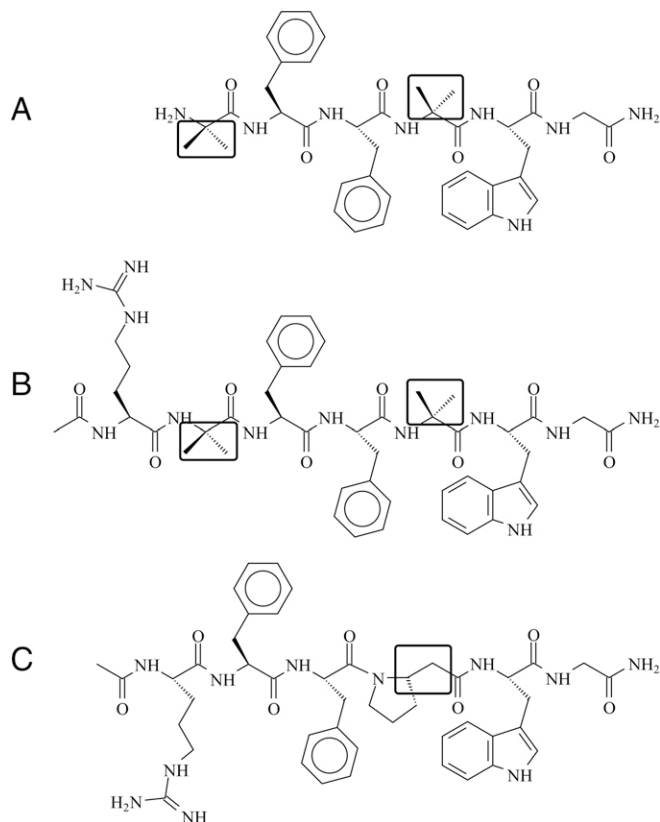


Fig. S1. Comparative structures of the three biostable insect kinin analogs: K-Aib-1 (1728) (*A*), K-Aib-3 (1729) (*B*) (12), and K- β A-1 (1460) (*C*) (26). The boxes highlight the unnatural portions of the peptide structures. In *A* and *B*, the boxes highlight the side chains of the bulky Aib residues. In *C* the box highlights the β -amino acid β^3 Pro that replaces the Pro that normally resides at that position in many natural insect kinins. Both *B* and *C* also feature an acetyl group (Ac) that caps the N terminus, located at the very left of the structures. This acetyl group adds further biostability against hydrolysis by aminopeptidases.

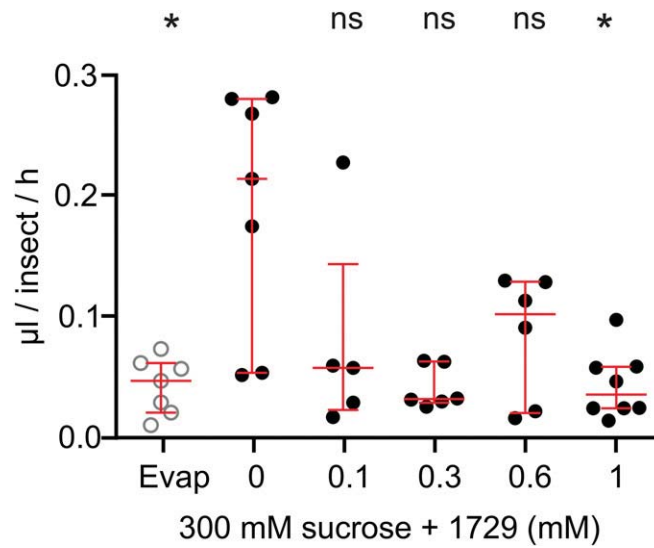


Fig. S2. Consumption of sugar mixed with kinin 1729 ingested by *A. aegypti* females during a 2-h CAFE assay. Groups of five females were starved for 48 h and then were exposed to a 5- μ L capillary tube containing 300 mM sucrose and kinin 1729. The volume of liquid that disappeared during the experiment was measured. Data show the individual measures (dots) and the median volume \pm first quartile (red lines) of five to eight replications. Ordinates: volume expressed in microliters per insect per hour. Abcissa: concentration of analog 1729 (0 to 1 mM) in a 300-mM sucrose solution; Evap, loss of sugar solution from vials without mosquitoes. Kruskal–Wallis ANOVA was followed by Dunn’s multiple comparison test (* $P < 0.05$); ns, not significant.

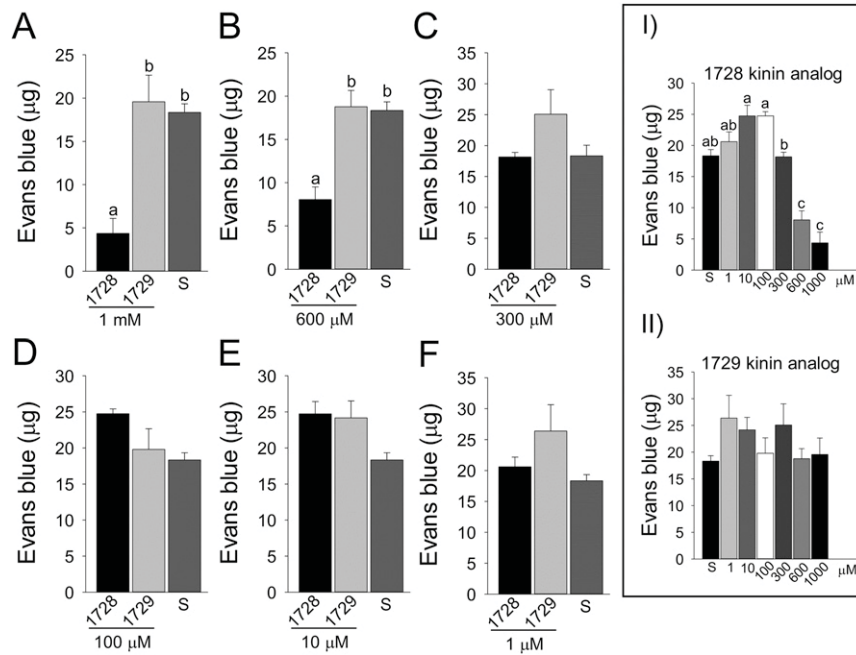


Fig. S3. Volume of Evans blue (expressed in micrograms) found in females exposed to kinin analogs in sucrose solution with Evans blue in no-choice feeding assays. After 5 h of dietary exposure to analogs at different concentrations or to control sucrose solution (S), the amount of Evans blue was measured from pooled females in each treatment. Three biological replicates were done per treatment. (A–F) Analogs 1728 and 1729 are compared at the same concentrations. (Inset I) Females exposed to 600 and 1,000 μ M of analog 1728 contained significantly less dye ($P < 0.01$). (Inset II) Analog 1729 did not have any effect on the residual amount of Evans blue, which at all analog concentrations was similar to that in mosquitoes exposed to the sucrose control. The data from three independent replications in each panel were analyzed with one-way ANOVA followed by Tukey’s HSD test. In A and B, bars represent mean \pm SEM. Common letters indicate nonsignificant differences at $P < 0.05$. (C–F). No significant differences were found among diets. (Insets) The data in A–F were analyzed with one-way ANOVA followed by Tukey’s HSD test but comparing the different concentrations of the same analog. Bars represent the mean \pm SEM. (Inset I). A common letter indicates no significant difference; $P < 0.05$. (Inset II) No significant difference was observed. One-way ANOVA was performed after an initial univariate GLM analysis demonstrated there was a significant effect of analogs and their concentrations that influenced Evans blue content ($P < 0.0001$).

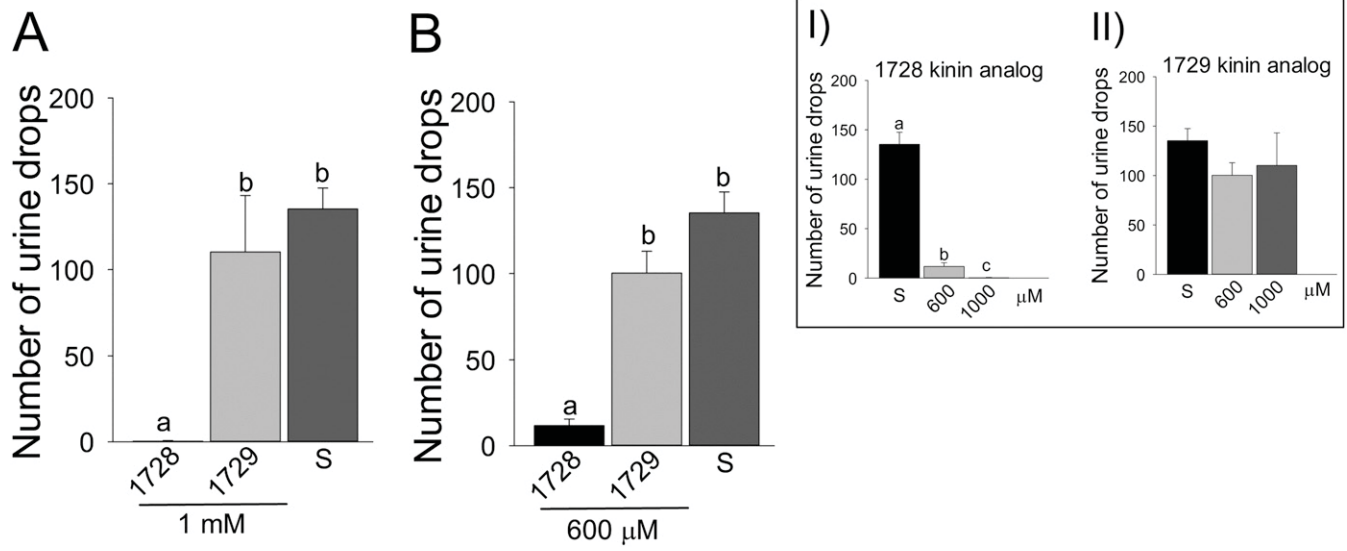


Fig. 54. Analog 1728 significantly decreases the number of urine drops excreted by females in no-choice feeding assays. (A and B) The total numbers of urine drops excreted by females exposed to analogs 1728 or 1729 at two concentrations of 1 mM or 600 mM or to control sucrose solution (S) were counted after 5 h of dietary exposure. (Insets) The data shown in A and B were analyzed to compare the effect of different concentrations of the same analog (Inset I, 1728; Inset II, 1729). No significant differences were found among sucrose and concentrations of analog 1729 (Inset II). The data were analyzed with nonparametric ANOVA and a Kruskal–Wallis test. Bars represent mean ± SEM, and common letters indicate no significant differences at $P < 0.05$ (Inset I).

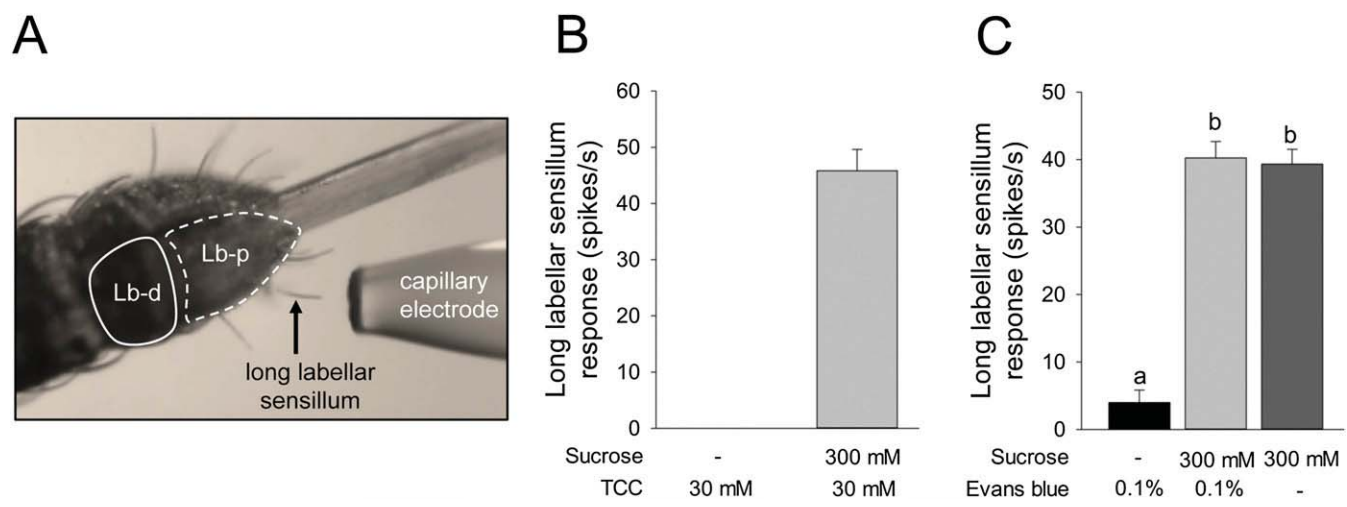


Fig. 55. (A) Photograph showing long labellar sensilla from which recordings were performed. A stimulus capillary electrode is positioned to cover one sensillum (arrow). Details are described in *SI Materials and Methods*. Lb-d, labellar distal segment; Lb-p, labellar proximal segment. (B) Responses of sweet-taste neurons in a single *A. aegypti* female labellar long hair sensillum to TCC electrolyte alone (30 mM) or sucrose solution (300 mM) with TCC. TCC as a control did not trigger spiking activity. (C) Responses (the number of spikes in the first second of a 2-s recording) of *A. aegypti* female labellar long hair sensillum to solutions of 0.1% Evans blue without or with sucrose (300 mM) or to sucrose alone (300 mM). The response to 0.1% Evans blue was insignificant, and the number of spikes per second did not differ in the other two treatments (300 mM sucrose + 0.1% Evans blue and 300 mM sucrose alone). One-way ANOVA with Tukey test; $P < 0.05$. Eight females were used.

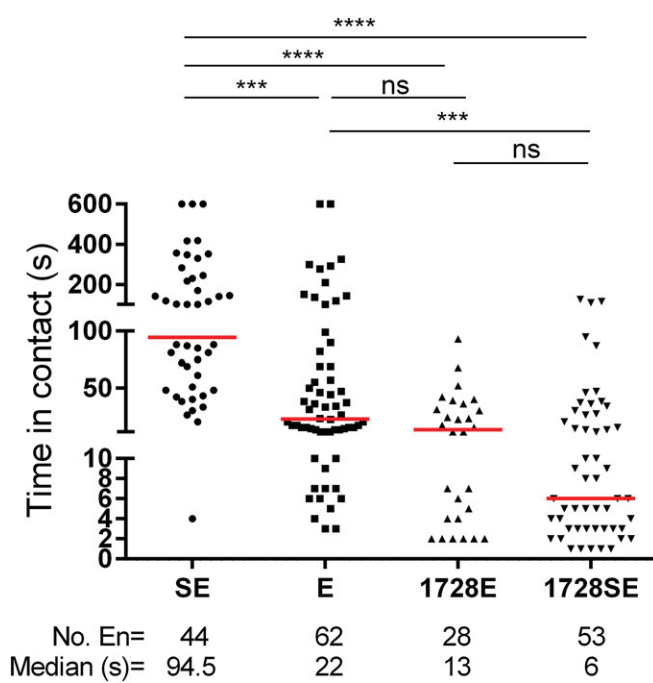


Fig. S6. Time (in seconds) female mosquitoes spent in contact with solutions of sucrose with Evans blue (SE), Evans blue only (E), kinin analog 1728 at 1 mM with Evans blue and without sucrose (1728E), or kinin analog 1728 at 1 mM with Evans blue and with sucrose (1728SE). The total number of encounters (En) and the median time are shown below each column. Data were analyzed by Kruskal–Wallis nonparametric ANOVA followed by Dunn’s multiple comparisons test. Asterisks denote significant differences ($***P < 0.001$, $****P < 0.0001$); ns, not significant.

<i>Aedae</i> KR-FLb	MRAVDGIAFHYANNNTLNGSDVEIVKEQDALYDVPVGLVLLSIFYGTISI IAVI	55
<i>Aedae</i> KR-FL	MRAVDGIAFHYANNNTLNGSDVEIVKEQDALYDVPVGLVLLSIFYGTISI IAVI	55
<i>Aedae</i> KR-ML	MRAVDGIAFHYANNNTLNGSDVEIVKEQDALYDVPVGLVLLSIFYGTISI IAVI	55
<i>Aedae</i> KR-FMT	MRAVDGIAFHYANNNTLNGSDVEIVKEQDALYDVPVGLVLLSIFYGTISI IAVI	55
<i>Aedae</i> KR-FLb	GNSLVIWIVLTTKQMQTITNMFIANLALADVTIAVFAIPFQQAAVLQRWNLPEF	110
<i>Aedae</i> KR-FL	GNSLVIWIVLTTKQMQTITNMFIANLALADVTIAVFAIPFQQAAVLQRWNLPEF	110
<i>Aedae</i> KR-ML	GNSLVIWIVLTTKQMQTITNMFIANLALADVTIAVFAIPFQQAAVLQRWNLPEF	110
<i>Aedae</i> KR-FMT	GNSLVIWIVLTTKQMQTITNMFIANLALADVTIAVFAIPFQQAAVLQRWNLPEF	110
<i>Aedae</i> KR-FLb	MCPFCPFVQLLSVNVSVFTLTAIAVDRHRAIINPLRARASKNISKFVISAIWMS	165
<i>Aedae</i> KR-FL	MCPFCPFVQLLSVNVSVFTLTAIAVDRHRAIINPLRARASKNISKFVISAIWMS	165
<i>Aedae</i> KR-ML	MCPFCPFVQLLSVNVSVFTLTAIAVDRHRAIINPLRARASKNISKFVISAIWMS	165
<i>Aedae</i> KR-FMT	MCPFCPFVQLLSVNVSVFTLTAIAVDRHRAIINPLRARASKNISKFVISAIWMS	165
<i>Aedae</i> KR-FLb	FALAAPTFLFALRVVPSIVSLGETNETYINMTKPFQVNVFESEMLLYRYILTTL	220
<i>Aedae</i> KR-FL	FALAAPTFLFALRVVPSIVSLGETNETYINMTKPFQVNVFESEMLLYRYILTTL	220
<i>Aedae</i> KR-ML	FALAAPTFLFALRVVPSIVSLGETNETYINMTKPFQVNVFESEMLLYRYILTTL	220
<i>Aedae</i> KR-FMT	FALAAPTFLFALRVVPSIVSLGETNETYINMTKPFQVNVFESEMLLYRYILTTL	220
<i>Aedae</i> KR-FLb	VQYFVPLCVISFVYIQMALRLWGSKTPGNAQDSRDMTMLKNKKVKMLIIVVAL	275
<i>Aedae</i> KR-FL	VQYFVPLCVISFVYIQMALRLWGSKTPGNAQDSRDMTMLKNKKVKMLIIVVAL	275
<i>Aedae</i> KR-ML	VQYFVPLCVISFVYIQMALRLWGSKTPGNAQDSRDMTMLKNKKVKMLIIVVAL	275
<i>Aedae</i> KR-FMT	VQYFVPLCVISFVYIQMALRLWGSKTPGNAQDSRDMTMLKNKKVKMLIIVVAL	275
<i>Aedae</i> KR-FLb	FGICWFPPLQLYNILHVTWSEVNEYRYINIIWFVCDWLAMNSCYNPFYGIYNEK	330
<i>Aedae</i> KR-FL	FGICWFPPLQLYNILHVTWSEVNEYRYINIIWFVCDWLAMNSCYNPFYGIYNEK	330
<i>Aedae</i> KR-ML	FGICWFPPLQLYNILHVTWSEVNEYRYINIIWFVCDWLAMNSCYNPFYGIYNEK	330
<i>Aedae</i> KR-FMT	FGICWFPPLQLYNILHVTWSEVNEYRYINIIWFVCDWLAMNSCYNPFYGIYNEK	330
<i>Aedae</i> KR-FLb	EKREFHKRYPPFRGRVQSYHQEQLTDKTLSMFTRVSSIRSNYATSSIRNKLYTGPI	385
<i>Aedae</i> KR-FL	EKREFHKRYPPFRGRVQSYHQEQLTDKTLSMFTRVSSIRSNYATSSIRNKLYTGPI	385
<i>Aedae</i> KR-ML	EKREFHKRYPPFRGRVQSYHQEQLTDKTLSMFTRVSSIRSNYATSSIRNKLYTGPI	385
<i>Aedae</i> KR-FMT	EKREFHKRYPPFRGRVQSYHQEQLTDKTLSMFTRVSSIRSNYATSSIRNKLYTGPI	385
<i>Aedae</i> KR-FLb	GGGSGNGGTHVGSYSSAFYQNQNSHHQOSYKSPNTNSAAGYQRNSTTDRNSSR	440
<i>Aedae</i> KR-FL	GGGSGNGGTHVGSYSSAFYQNQNSHHQOSYKSPNTNSAAGYQRNSTTDRNSSR	440
<i>Aedae</i> KR-ML	GGGSGNGGTHVGSYSSAFYQNQNSHHQOSYKSPNTNSVAGYQRNSTTDRNSSR	440
<i>Aedae</i> KR-FMT	GGGSGNGGTHVGSYSSAFYQNQNSHHQOSYKSPNTNSVAGYQRNSTTDRNSSR	440
<i>Aedae</i> KR-FLb	KTAAGAPWDPKCCPCRONSTRTSTAAASACPYRMPLPAVASDGDGSGSEGGPNSA	495
<i>Aedae</i> KR-FL	KTAAGAPWDPKCCPCRONSTRTSTAAASACPYRMPLPAVASDGDGSGSEGGPNSA	495
<i>Aedae</i> KR-ML	KTAAGAPWDPKCCPCRONSTRTSTAAASACPYRMPLPAVASDGDGSGSEGGPNSA	495
<i>Aedae</i> KR-FMT	KTAAGAPWDPKCCPCRONSTRTSTAAASACPYRMPLPAVASDGDGSGSEGGPNSA	495
<i>Aedae</i> KR-FLb	GGGQSPMINNDRQLLGADDNYGSAAQKLEVISLDHPPDSADENGVAETLHSR	550
<i>Aedae</i> KR-FL	GGGQSPMINNDRQLLGADDNYGSAAQKLEVISLDHPPDSADENGVAETLHSR	550
<i>Aedae</i> KR-ML	GGGQSPMINNDRQLLGADDNYGSAAQKLEVISLDHPPDSADENGVAETLHSR	550
<i>Aedae</i> KR-FMT	GGGQSPMINNDRQLLGADDNYGSAAQKLEVISLDHPPDSADENGVAETLHSR	550
<i>Aedae</i> KR-FLb	TANBOEQDERLQLTSFISSGNRHERFHHINNL	584
<i>Aedae</i> KR-FL	TANBOEQDERLQLTSFISSGNRHERFHHINNL	584
<i>Aedae</i> KR-ML	TANBOEQDERLQLTSFISSGNRHERFHHINNL	584
<i>Aedae</i> KR-FMT	TANBOEQDERLQLTSFISSGNRHERFHHINNL	584

Fig. S7. Alignment of *Aedae*-KR amino acid sequences translated from full-length ORF cDNAs from female appendages (labellum and legs) (this work) and Malpighian tubules (GenBank AAT95982) (9) and from male legs (this work). Identical residues are shaded in black. The stretch of sequences shaded in gray indicates the antigenic sequence used for anti-peptide antibody production (5, 7). In cDNAs from female appendages, there were nonsynonymous SNPs with respect to the Malpighian tubule sequence, resulting in changes in amino acid residues for the protein expressed in labellum and legs in females, indicated as follows: Malpighian tubule residue-residue position-appendage residue: Gly277Glu and Asp355Gly in female labellum and Ser411Pro in female legs. In both appendages in females, the following substitutions were found: Asn403Ser, Val425Ala, Cys492Tyr, Asp539Glu, Glu540Gly, Pro547Leu, Gly554Glu, and Phe579Leu. Substitutions found in male legs were Val221Ala and Pro547Leu. These changes likely reflect allelic variations in the individuals from which the Malpighian tubule cDNA was originally cloned (9). FL, female legs; FLb, female labellum; FMT, female Malpighian tubules; ML, male legs.

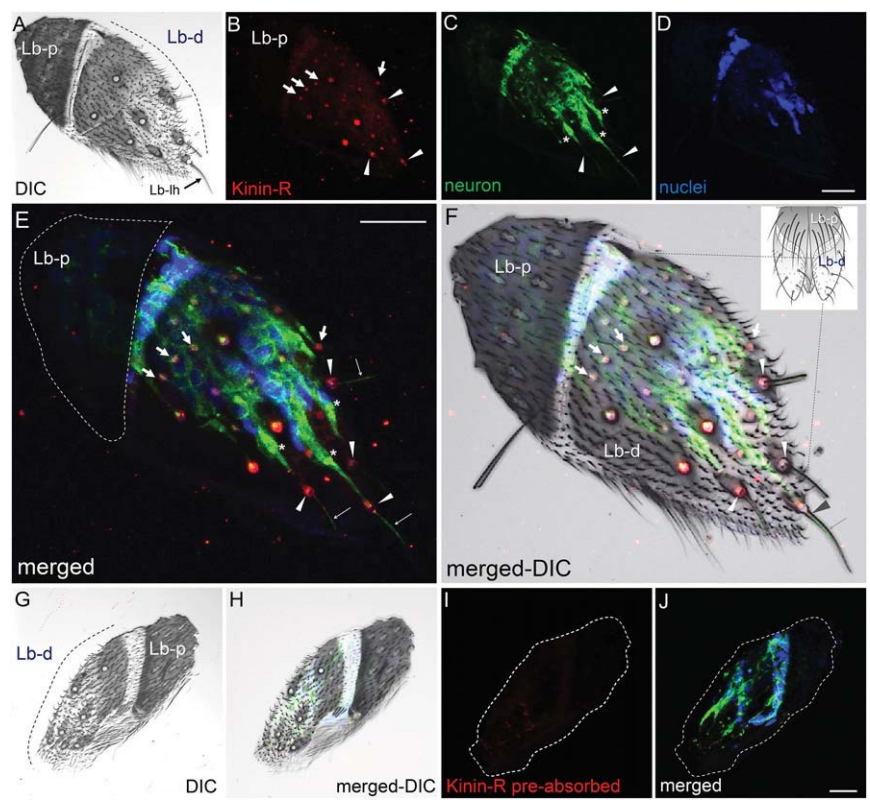


Fig. 58. Confocal analyses of *Aedae*-KR immunolocalization in the distal segment of the labellum. In A, F, G, and H, DIC images show the labellum is composed of two segments, proximal and distal (dashed black lines in A, F, and G), where long labellar sensilla (long hair, black arrow in A) are present (see drawing in F). In C, E, F, H, and J neurons appear green (anti-HRP antibody). In D, E, F, H, and J nuclei appear blue (DAPI). The root fiber bundles in the ciliary region of sensory neurons are marked by asterisks in C and E. The receptor signal (red) is present in accessory cells at the base of long labellar sensilla, indicated by arrowheads in B, E, and F, and in accessory cells of short papilla, indicated by arrows in B, E, and F. No receptor signal was observed in the labellum proximal segment, the area enclosed by the dashed white line in E. Dendrites projecting toward the tip of the long labellar hair are shown in E and F (thin arrows). No receptor signal was observed in negative control tissues (antigen preabsorbed antibodies). G–J show the same tissue. Images as Z-stacks (Z-step: 0.66 μm) are as follows: A–F, 12 sections; G–J, 10 sections. Lb-d, distal segment of labellum; Lb-lh, long labellar hair; Lb-p, proximal segment of labellum. (Scale bars, 20 μm.)

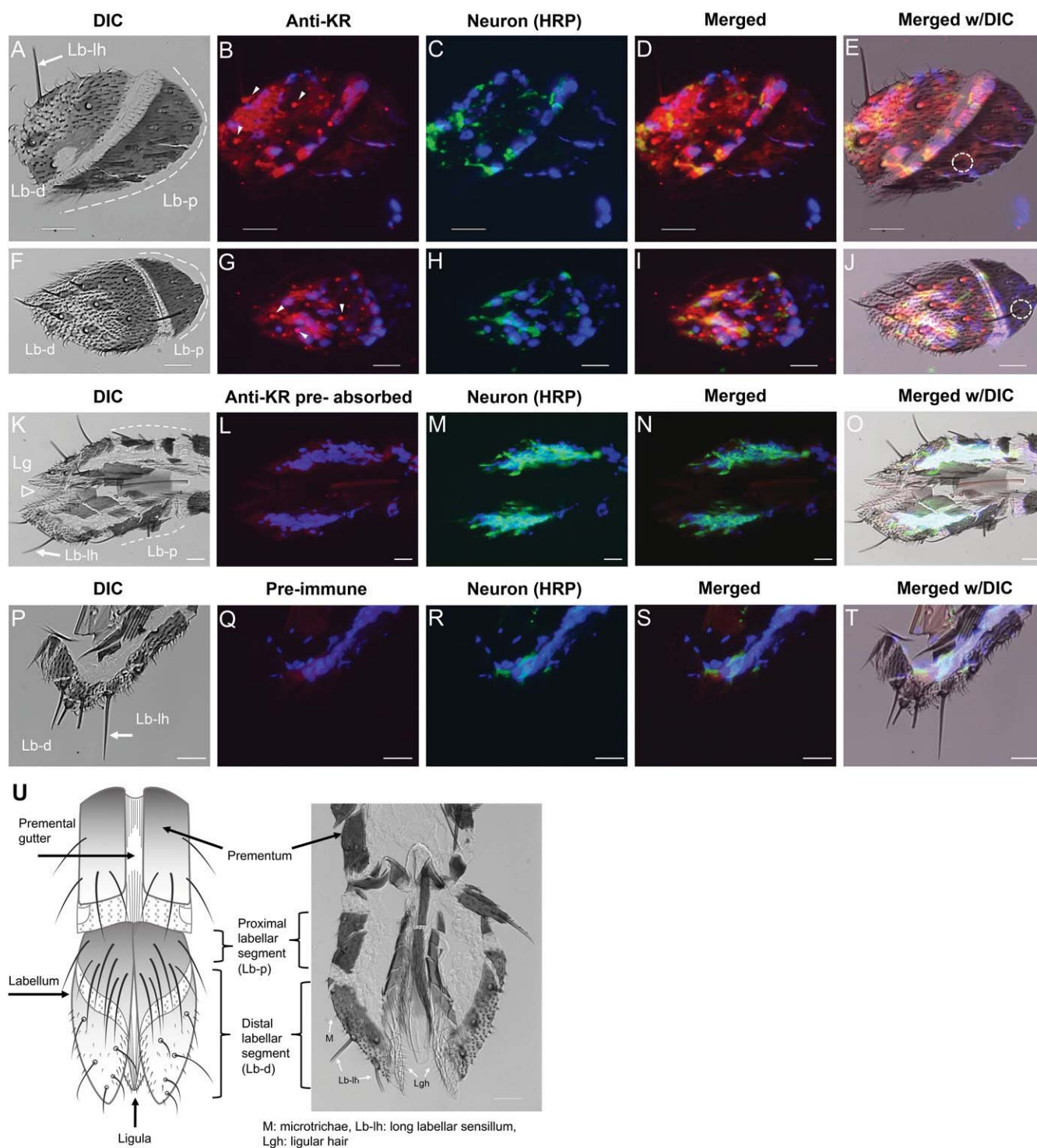


Fig. S9. Immunofluorescence analyses of the *Aedae-KR* in female labellum. (A, F, K, and P) DIC images show the distal and proximal (dashed white lines) labellar segments and ligula (open arrowhead in K). (B and G) The receptor signal (red) was observed in sensory neurons of labellar sensilla (arrowheads) only in the distal labellar segment. (E and J) No receptor signal was observed in the proximal labellar segment, although hairs/sensilla were present (dashed circle). (L, N, O, Q, S, and T) No receptor signal was observed in tissues incubated with antigen preabsorbed antibody (L, N, and O) or with preimmune serum (Q, S, and T). In C, H, M, and R, neurons appear green. Merged images of receptor, neuron, and nuclear labeling (blue; DAPI) are shown in D, I, N, and S. Merged DIC images are shown in E, J, O, and T. (U) A schematic diagram of the labellum (28, 33) is compared with a labellar frozen section. Lb-d, distal segment of labellum; Lb-lh, long labellar hair; Lb-p, proximal segment of labellum; Lg, ligula; Lgh, ligular hair; M, microtrichae. (Scale bars, 20 μ m.)

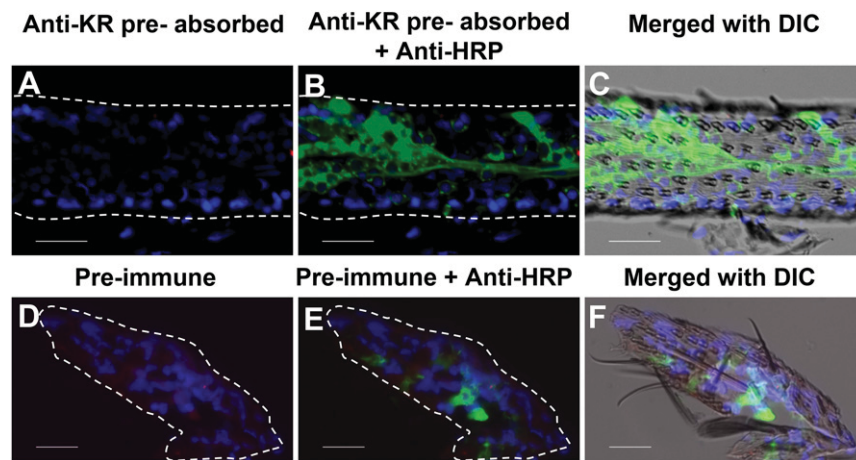


Fig. S10. Negative controls for the immunolocalization of *Aedae*-KR in female prothoracic tarsal sensory neurons. Neurons appear in green (anti-HRP). No receptor signal in tarsi was observed with antigen-preabsorbed anti-KR-Ct₃₂₈₋₃₄₅ antibody (A–C) or preimmune antibody (D–F). (Scale bars, 20 μm.)

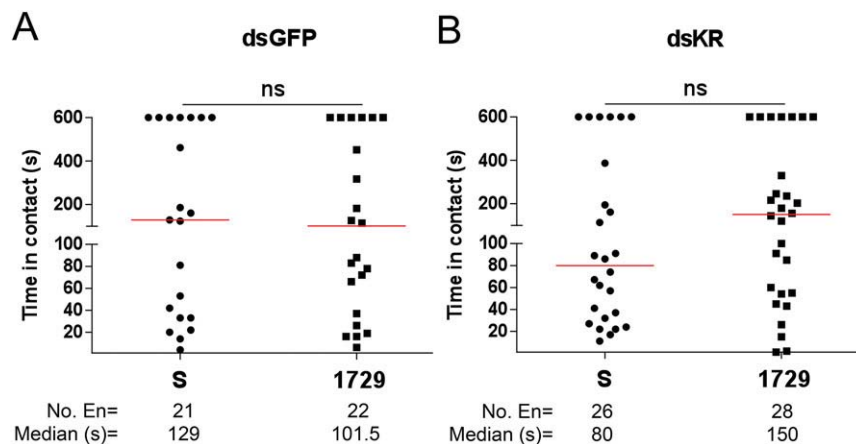


Fig. S11. There is no difference in the median time spent in contact with sucrose solution or kinin analog 1729 at a concentration of 1 mM by females treated with dsGFP (A) or dsKR (B). Data were analyzed using a Mann–Whitney test using GraphPad Prism. Three independent replicates were performed. ns, not significant.

Table S1. Primers used for cloning and RNAi of *Aedae*-KR

Primers	Primer sequences
Primers for PCR	
<i>Aedaekinin</i> 5' UTR 0	5'-GGAATACCAAATTATCTACAAGAATGCGA-3'
<i>Aedaekinin</i> 3' UTR 1	5'-TGGTTCAAATTCACAAGTTGTTAATGTGC-3'
<i>Aedaekinin</i> F2	5'-AACGAGAATCCACAAGCGATATCCGTTC-3'
<i>Aedaekinin</i> R2	5'-AAGTCCGGTAGAGTTTGGCCGACATGG-3'
<i>Aedaekinin</i> F3	5'-CAAGTGTGTCCATGTCGGCAAACTCTACC-3'
<i>Aedaekinin</i> R3	5'-GGAACGGATATCGCTTGTGGAATTCCTCG-3'
<i>Aedaekinin</i> 5'UTR	5'-TTAGCTGTCGTATGTATCAACACGGATCATTG-3'
<i>Aedaekinin</i> 3'UTR0	5'-GATTTTCTCATTAACTAGGGTAAGTGTACC-3'
<i>Aedaekinin</i> 5'UTR0	5'-GGAATACCAAATTATCTACAAGAATGCGA-3'
<i>Aedaekinin</i> 3'UTR1	5'-TGGTTCAAATTCACAAGTTGTTAATGTGC-3'
Primers for syntheses of dsRNA	
<i>Aedaekinin</i> T7F	5'-TAATACGACTCACTATAGGGTGTATCAACACGGATCATTGCATGGAATGGC-3'
<i>Aedaekinin</i> T7R	5'-TAATACGACTCACTATAGGGCATCGCTGCCGTTTCAGTGTATTGTTGTTGC-3'
GFPT7F	5'-TAAATACGACTCACTATAGGGAGAATGGTGAGCAAGGGCGAGAGCTGT-3'
GFPT7R	5'-TAAATACGACTCACTATAGGGAGATTACTTGTACAGCTCGTCCATGCC-3'
Primers for qPCR	
<i>Aedaekinin</i> QPCRf	5'-CGAGAATCCACAAGCGATA-3'
<i>Aedaekinin</i> QPCRr	5'-ACTGCTCCTGGTGGTAGCTT-3'
<i>Aedae</i> 18SRQPCRf	5'-CCTTCAACAAGGATCAAGTGG-3'
<i>Aedae</i> 18SRQPCRr	5'-GGAGTAGCACCCGTGTTGG-3'

Underlined sequences indicate T7 polymerase promoter.

Movie S1

Females of *A. aegypti* feeding on
kinin analog 1728 at 1 mM
(10% sucrose solution, 0.1% Evans blue)

Movie S1. Video recordings (montage) of female mosquitoes' behavior during the first hour of exposure to kinin analog 1728 at 1 mM in 10% sucrose solution with Evans blue (0.1%). Fast aversive behavior (walking, flying, or jumping away) was observed shortly after females touched the diets with their legs and/or probed them with their proboscis; the median time spent in contact was 6 s (Fig. 1).

[Movie S1](#)

Movie S2

Females of *A. aegypti* feeding on
Sucrose solution
(10% sucrose, 0.1% Evans blue)

Movie S2. Video recordings of female mosquito behavior when contacting the 10% sucrose solution with Evans blue (0.1%). Three females fed on the sucrose solution, remaining in contact for several seconds; the median time spent in contact was 94.5 s.

[Movie S2](#)

E. Article 5 : inhibition sugar response by A leucokinin in *Drosophila melanogaster*.

en préparation

Abstract

F. Article 6 : Immediate perception of a reward is distinct from the reward's long-term salience .

[eLife](https://doi.org/10.7554/eLife.22283) 2016 Decembre 22. eLife 2016;10.7554/eLife.22283. <http://dx.doi.org/10.7554/eLife.22283>

Abstract

Reward perception guides all aspects of animal behavior. However, the relationship between the perceived value of a reward, the latent value of a reward, and the behavioral response remains unclear. Here we report that, given a choice between two sweet and chemically similar sugars-L- and D-arabinose-*Drosophila melanogaster* prefers D- over L-arabinose, but forms long-term memories of L-arabinose (the isomer present in ripening fruits) more reliably. Behavioral assays indicate that L-arabinose-generated memories require sugar receptor Gr43a, and calcium imaging and electrophysiological recording indicate that L- and D-arabinose differentially activate Gr43a-expressing neurons. We posit that the immediate valence of a reward is not always predictive of the long-term reinforcement value of that reward, and that a subset of sugar-sensing neurons may generate distinct representations of similar sugars, allowing for rapid assessment of the salient features of various sugar rewards and generation of reward-specific behaviors. However, how sensory neurons communicate information about L-arabinose quality and concentration-features relevant for long-term memory-remains unknown.

Immediate perception of a reward is distinct from the reward's long-term salience

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Abstract Reward perception guides all aspects of animal behavior. However, the relationship between the perceived value of a reward, the latent value of a reward, and the behavioral response remains unclear. Here we report that, given a choice between two sweet and chemically similar sugars—L- and D-arabinose—*Drosophila melanogaster* prefers D- over L- arabinose, but forms long-term memories of L-arabinose more reliably. Behavioral assays indicate that L-arabinose-generated memories require sugar receptor Gr43a, and calcium imaging and electrophysiological recordings indicate that L- and D-arabinose differentially activate Gr43a-expressing neurons. We posit that the immediate valence of a reward is not always predictive of the long-term reinforcement value of that reward, and that a subset of sugar-sensing neurons may generate distinct representations of similar sugars, allowing for rapid assessment of the salient features of various sugar rewards and generation of reward-specific behaviors. However, how sensory neurons communicate information about L-arabinose quality and concentration—features relevant for long-term memory—remains unknown.

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Introduction

In an environment filled with various stimuli, the positive experiences an animal remembers are widely assumed to be rewarding and salient. Long-term associative memories in particular are supposed to reflect the intensity of past responses to rewards. The experiences we remember, however, are not always those we expect to remember. How immediate reward perceptions influence future actions is therefore of wide interest.

Among various positive rewards, food, and in particular sweet food, has been most revealing since it is a source of both pleasure (immediate value) and nutrition (long-term value). Food is also a complex reward. Having evolved in distinct ecological niches, different species of *Drosophila* display distinct food preferences and discriminate between potential sources of nutrition (*Dethier, 1976*). For example, while some species of *Drosophila* prefer rotting fruits, others prefer mushrooms, cacti, or hibiscus flowers (*Markow and O'Grady, 2005*). Identifying and remembering relevant food, therefore, is essential for survival. Moreover, food is often not a single substance but a mixture of various compounds, and not all are equally rewarding: rotting fruits contain various sugars, alcohols, and acids that produce varying responses (*Yarmolinsky et al., 2009; Charlu et al., 2013*). Food in

eLife digest We often remember experiences that are rewarding in some way. However, not every rewarding experience is stored in memory, and the particular experiences we remember are not always those we would expect to remember. Why is it that some experiences generate long-term memories whereas others do not?

Fruit flies feed on a variety of different sugars present in rotting fruits. Although the flies find all of these sugars attractive, they form memories of some sugars more readily than others. This distinction is particularly striking in the case of two sugars with similar structures: D-arabinose and L-arabinose. Flies typically prefer D-arabinose over L-arabinose, but are more likely to remember an encounter with L-arabinose than D-arabinose.

McGinnis et al. have used fruit flies to explore how the rewarding properties of an experience affect how likely it is to be stored in memory. The experiments show that D-arabinose and L-arabinose generate different patterns of activity in the fly brain, and identify a subset of taste neurons that support the formation of memories specifically about L-arabinose. These neurons enable flies to associate features of their environment – such as odors – with the presence of this one particular sugar. Such memories may help the flies to find a similar food source again in the future. Artificially activating these neurons is also sufficient to trigger the formation of a memory, even in the absence of L-arabinose itself.

Taken as a whole, this work demonstrates that the immediate appeal of a reward can be separated from its ability to generate a long-term memory. The fact that activation of taste neurons can trigger memory formation explains how flies can quickly form long-term memories about desirable food sources. Looking ahead, further work will be required to understand the mechanisms that determine what animals like at any given moment, and what they remember over time.

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natural contexts is also always part of an environment filled with other features, including predators, and therefore quick evaluation of potential food sources requires simultaneous processing of multiple stimuli. Finally, the attraction to food, and memories of it, are influenced by the internal state of the organism, such as whether the animal is hungry or satiated (*Colomb et al., 2009; Krashes et al., 2009; Toshima and Tanimura, 2012; Dethier, 1976*). It is therefore likely that contingent on their internal state, animals use certain components of food sources to quickly recognize those that are appropriate for feeding and, if worthwhile, to form memories of these sources for future visits. How these different aspects of food very quickly generate appropriate memories that guide future food-seeking behavior, however, remains unclear.

One possibility is that whatever components of food are most salient for long-term behavior are the same features that animals find immediately rewarding. This would predict that the more appealing (or palatable) a sugar is, the better it will be remembered. Another possibility is that certain components of food can reinforce memory relatively independent of the food's immediate appeal, because they indicate specific attributes of the food (e.g. nutritional content) that are of long-term relevance. In a complex environment, where an animal needs to process multiple stimuli simultaneously, such processing may ensure that regardless of the immediate response, stimuli of long-term relevance will be remembered.

In the course of exploring both the immediate appeal of various natural sugars and their ability to generate long-term associative memories, we serendipitously discovered that these two processes are separable. A specific illustration of this phenomenon is seen with the two chemically similar sugars, D- and L-arabinose: flies greatly prefer D-arabinose to L-arabinose, but better remember an odor paired with L-arabinose than with D-arabinose. We have also begun to explore how an animal assesses whether an experience that is rewarding in the moment is also of long-term relevance. Many studies have characterized higher order systems, particularly the neuromodulatory systems such as dopaminergic (*Schwaerzel et al., 2003; Huetteroth et al., 2015; Berry et al., 2012; Liu et al., 2012; Yamagata et al., 2015; Musso et al., 2015*), octopaminergic (*Burke et al., 2012; Schwaerzel et al., 2003*), neuropeptide F (*Krashes et al., 2009*) and mushroom body neurons (*Aso et al., 2014; Kirkhart and Scott, 2015; Vogt et al., 2014*) underlying long-term sugar reward

memory in *Drosophila*. How various sugars differentially engage the higher order reward system, however, remains unclear. We find that D- and L-arabinose differentially activate the same peripheral Gr43a-expressing neurons, and that activating Gr43a in some but not all manners can substitute for the sugar reward, indicating that sensory neurons can at least partially mediate this discrimination process. However, the exact mechanism by which these sensory neurons communicate the relevant features of L-arabinose to higher order systems remains unclear at this stage.

Results

***Drosophila melanogaster* prefers D-arabinose but more reliably forms long-term memories of odors paired with L-arabinose**

To explore how animals evaluate salient features of food, we used an associative-appetitive memory paradigm (henceforth referred to as the 'memory paradigm') with *Drosophila melanogaster* that approximates food-seeking behavior (Colomb *et al.*, 2009; Krashes and Waddell, 2011; Tempel *et al.*, 1983). In this paradigm, hungry flies are trained for 2 min to associate an odor with a rewarding sweet sugar; trained flies subsequently seek out the sugar-associated odor for several days afterwards, indicating that they have formed an associative memory (Figure 1A). We have used this paradigm for three reasons: one, it is an ethologically relevant behavior; two, both the internal state (hunger) of the fly and characteristics of the sugar dictate the duration of memory (Burke and Waddell, 2011; Fujita and Tanimura, 2011; Colomb *et al.*, 2009); and three, salient features of the sugar are evaluated rapidly within the 2-min training as reported by others (Burke and Waddell, 2011) and similarly confirmed by us (Figure 1—figure supplement 1A and B).

In the course of training flies (Figure 1A) with various sugars, including those that are present in *Drosophila melanogaster*'s natural diet of ripening fruits (Figure 1B) some of which are non-nutritious (Figure 1C), we observed that the relative appeal of a sugar (preference, Figure 1D) or short-term memory (minutes after training, Figure 1E) does not always predict its ability to act as a rewarding stimulus for long-term (24 hr after training) associative memory (Figure 1F and Figure 1—figure supplement 1C). This was apparent for multiple sugars, but nowhere so striking as the difference between two structural isomers, D- and L-arabinose (Figure 2A). D- and L-arabinose both taste sweet (Figure 2—figure supplement 1) and are both non-nutritious (Figure 2B). Flies overwhelmingly preferred D-arabinose to L- (Figure 1D and Figure 1—figure supplement 1C), and form similar short-term memories of both sugars (Figure 1E and Figure 2C). However, it is L-arabinose, not D-, that is more effective in producing long-term memory (Figure 1F and Figure 2C). The relative ineffectiveness of D-arabinose in producing long-term memory is consistent with other studies (Burke and Waddell, 2011; Fujita and Tanimura, 2011; Cervantes-Sandoval and Davis, 2012).

A trivial explanation for the observed memory with L-arabinose would be contamination with nutritious sugars. But L-arabinose bought from different sources generated similar survival curves and memory scores (Figure 2—figure supplement 2A). The L-arabinose memory is also not due to particular wild-type flies used in the experiment (Figure 2—figure supplement 2B), the training conditions or the particular experimenter (Figure 2—figure supplement 2C). Neither arabinogalactan (a polymer of L-arabinose and galactose) nor the natural L-sugar rhamnose, produce significant memory, indicating that not all L-arabinose-containing components of fruits' cell wall or natural L-sugars are conducive to memory formation (Figure 2—figure supplement 2D). Bacteria are known to utilize L-arabinose (Watanabe *et al.*, 2006), but the flies' resident bacteria had no evident contribution to L-arabinose memory, since giving the flies a cocktail of antibiotics for the 2 days prior to behavioral training had no effect on L-arabinose memory (Figure 2—figure supplement 2E). Taken together, these results suggest that L-arabinose can act as a rewarding stimulus for long-term associative memory.

We wondered whether the behavioral differences between D- and L- were due to the high concentration (1 M) of sugars, although 1 M to 3M sugar is standard in memory assays (Yamagata *et al.*, 2015; Cervantes-Sandoval and Davis, 2012; Burke and Waddell, 2011). However, the preference for D-arabinose (Figure 2D) persisted when the sugars' concentrations were both reduced 100-fold (10 mM), and began to shift only when the concentration of D-arabinose was reduced to less than a third of L-arabinose (Figure 2—figure supplement 3A). A similar difference between D- and L-arabinose has also been reported in the blowfly *Phormia regina*, where the taste

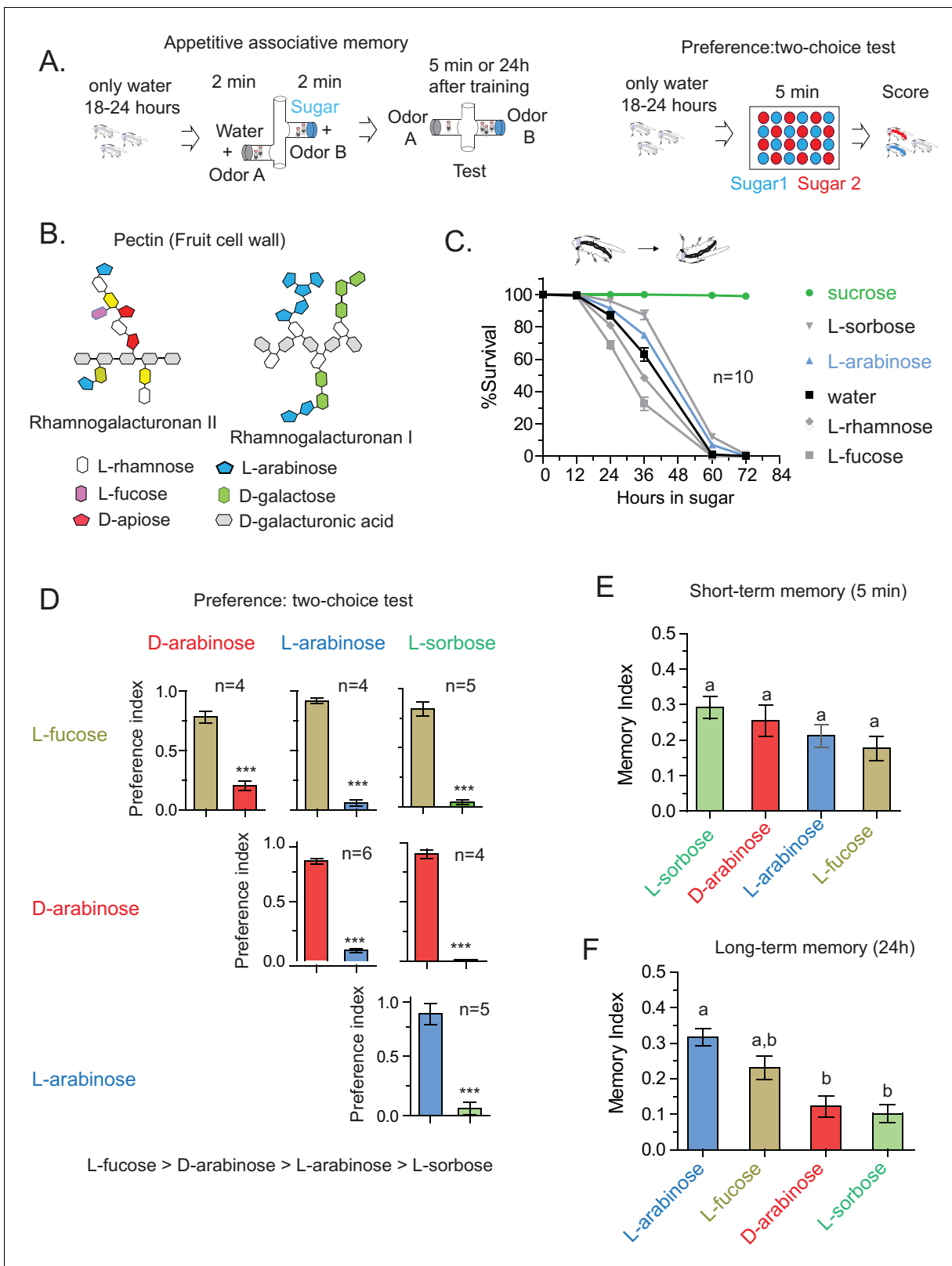


Figure 1. Flies' immediate preference for a sugar is not predictive of their long-term memory: various sugars from fruits. (A) Schematic of behavioral assays. In the appetitive associative memory paradigm, hungry flies are trained for 2 min with the sugar-odor pair and memory is assayed by subsequently giving a choice between the two odors. In the preference assay, hungry flies are given a choice between two sugars mixed with different colors; after 5 min color of the abdomen is used to assess consumption. (B) Schematic of selected pectic polysaccharides present in fruits' cell wall

Figure 1 continued on next page

Figure 1 continued

walls, adapted from Harholt et al. (**Harholt et al., 2010**). (C) Survival percentages for flies given solely 1 M sugar solutions. $n = 10$ (50 flies per n) for each time point. (D) Two-choice tests comparing flies' preference for each of four sugars when both sugars are presented side-by-side for 5 min (50 flies per n). (E) Short-term (5 min) associative memory scores for the sugars. ($n = 7$ –11) (F) Long-term (24 hr) associative memory scores for the sugars. L-fucose is a component of pectin as well, although the amount is low compared to L-arabinose. ($n = 20$ –24) Memory scores labeled *a* are significantly different (<0.05) from bars labeled *b*, analyzed by one way ANOVA with Tukey's multiple comparisons test. Detailed explanations of what constitutes a single n is found in Materials and methods. Results with error bars are means \pm s.e.m.

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The following figure supplement is available for figure 1:

Figure supplement 1. The CS-US association occurs during the two-minute training.

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threshold for D-arabinose is reported to be five times lower than that of L-arabinose (**Hassett et al., 1950**). When we measured consumption by Capillary Feeder (CAFÉ) assays over a concentration range (**Figure 2—figure supplement 3B**) or by mixing radioactive $[^{32}\text{P}]$ in the food in fixed concentration (**Figure 2—figure supplement 3C**), the flies consumed more D- than L-arabinose. Over time, however, flies consumed less D- and L-arabinose than nutritious sugars (data not shown), consistent with other studies (**Dus et al., 2011; Stafford et al., 2012**), and consumption reached a plateau in ~ 14 min for D- and ~ 30 min for L. We also monitored by video the behavior of single flies as they fed on colorless D- and L-arabinose solutions (**Figure 2—figure supplement 3D**) and observed that they spend much more time on D-arabinose than L-arabinose, consistent with higher overall consumption. These differences are not due to differences in mere detection of D- and L- arabinose: detection rates were very similar at high concentrations and began to differ only when concentrations were dropped to ≤ 50 mM (**Figure 2—figure supplement 1**). Likewise, the ability of L-arabinose to generate long-lasting memory persisted even at a 10-fold lower concentration, albeit with much weaker efficacy (**Figure 2E**). Moreover, lowering D-arabinose concentration, where the flies still detect D-arabinose but consume less of it, there was no increase in memory (**Figure 2—figure supplement 3E**) ruling out the possibility that consuming too much non-nutritious sugar, such as D-arabinose, is somehow a negative reinforcement.

In addition to consumption, we also measured the proboscis extension response (PER), which reports immediate acceptance of a taste stimuli. Curiously, PER response was similar between D- and L-arabinose over a concentration range (**Figure 2—figure supplement 4A**), consistent with other reports that PER depends more on the intensity than chemical nature of the sugar (**Masek and Scott, 2010; Stafford et al., 2012**). However, mere detection and acceptance of the sugar is not sufficient for long-term memory: A choice test between water and various concentrations of sucrose (a potent inducer of long-term memory) showed that there was no difference in the likelihood of consumption between 1 M and 10 mM sucrose; only when sucrose concentration is reduced to 1 mM did detection begin to fall (**Figure 2—figure supplement 4B**). However, only sucrose concentrations ≥ 100 mM reliably produced robust long-term memory (**Figure 2—figure supplement 4C**). Therefore, various sugar-associated behavioral responses, such as detection, acceptance, and assessment of immediate and long-term relevance are not a single process and are likely dictated by various attributes of the sugar. Taken together, these results suggest that even two chemically similar sugars can elicit quite distinct short- and long-term behaviors, and that immediate behavioral responses are not always predictive of long-term behavioral consequences: while flies find D-arabinose more immediately appealing, L-arabinose is more salient for long-term memory.

Gustatory neurons involved in L-arabinose memory and D-arabinose preference

What is the neural basis for the difference in behavioral responses to D- and L-arabinose? There are two possibilities, not mutually exclusive: the two sugars engage distinct neural pathways, or they activate the same neural pathways in a distinct manner. The initial step in sugar detection and consumption are the gustatory-receptor-expressing (Gr) neurons that respond to sweet substances. To date, Gr5a, Gr43a, Gr61a, and Gr64a, b, c, d, e, and f have been implicated in sweet sugar detection (**Dahanukar et al., 2007; Jiao et al., 2008; Yavuz et al., 2014; Freeman et al., 2014; Miyamoto et al., 2013**). We therefore used Gr-GAL4 drivers to express the inward rectifying

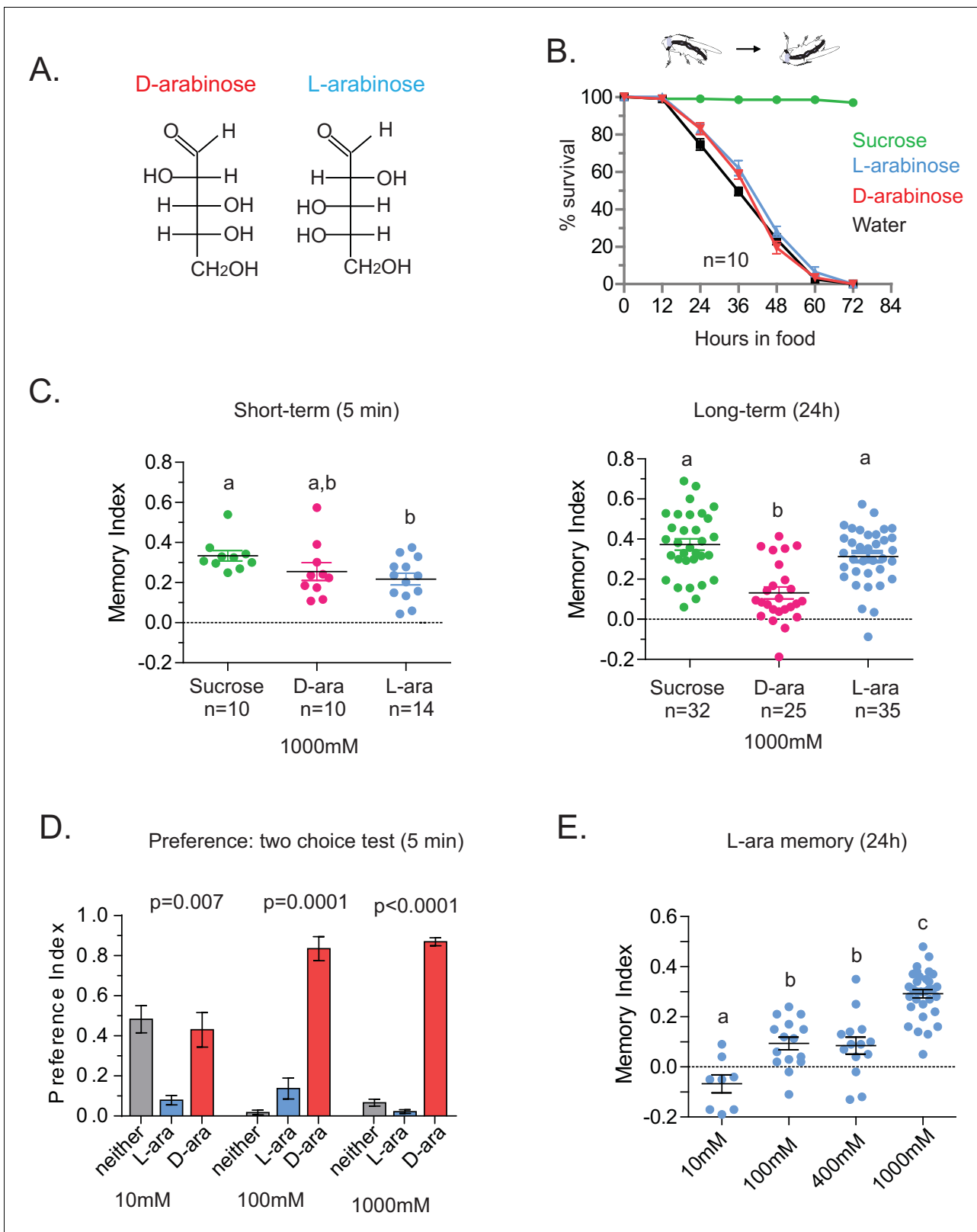


Figure 2. Flies' immediate preference for a sugar is not predictive of their long-term memory: L- vs D-arabinose. (A) Structures of D- and L-arabinose. (B) Survival percentages for flies given solely 1 M sugar solutions. $n = 10$ (50 flies per n) for each time point. (C) Short- and long-term memory of sucrose and D- and L-arabinose. (D) Two-choice tests comparing flies' preference for D- and L-arabinose when both sugars are presented side-by-side for 5 mins. $n = 4$ (50 flies per n). (E) Long-term (24 hr) memory scores for increasing concentrations of L-arabinose. Results with error bars are means \pm s.e.m. Figure 2 continued on next page

Figure 2 continued

ns, not significant. * ≤ 0.01 , ** ≤ 0.001 and *** ≤ 0.0001 . The significant differences ($p < 0.05$) between conditions in **Figure 2C and E** were analyzed by one-way ANOVA with Tukey's multiple comparisons test and differences are denoted by different letters. Detailed explanations of what constitutes a single n is found in Materials and methods.

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The following figure supplements are available for figure 2:

Figure supplement 1. Palatability of D- and L-arabinose over a concentration range.

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Figure supplement 2. Specificity of L-arabinose memory.

DOI: [10.7554/eLife.22283.007](https://doi.org/10.7554/eLife.22283.007)

Figure supplement 3. Although both are sweet, D-arabinose is preferred over L-arabinose.

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Figure supplement 4. Detection and memory.

DOI: [10.7554/eLife.22283.009](https://doi.org/10.7554/eLife.22283.009)

potassium channel Kir2.1 (Gr-GAL4/+; UAS-Kir2.1/+), silencing these sets of Gr-expressing neurons (**Baines et al., 2001**) in order to determine the neurons involved in D vs L preference and L-arabinose memory.

Preference and memory for a sugar starts with detecting the sugar; silencing neurons required for detection could cause a general decline in consumption of a particular sugar or all sugars. We therefore first measured the flies' ability to detect and consume L- or D-arabinose following silencing of specific Gr-expressing neurons (**Figure 3—figure supplement 1A and B**). Silencing Gr5a-expressing neurons reduced L-arabinose detection by about 80%, while silencing Gr61a-expressing neurons reduced both L- and D-arabinose detection by ~50%. Silencing Gr64e and Gr64f neurons almost completely abolished detection, discrimination and memory, consistent with previous reports that these receptors are likely expressed in all neurons responsible for sugar detection (**Jiao et al., 2008; Wisotsky et al., 2011**).

Silencing neurons required for discrimination would result in equivalent consumption of D- and L-arabinose. Upon silencing of Gr5a-, Gr43a-, Gr64a-, or Gr64d-expressing neurons, flies still overwhelmingly preferred D-arabinose (**Figure 3A**). Only silencing Gr61a neurons reduced D-arabinose consumption while increasing L-arabinose consumption (and ~30% flies did not eat any sugar), indicating that without Gr61a-expressing neurons flies were beginning to have trouble discriminating between the two sugars (**Figure 3A**).

In contrast to D-arabinose preference, silencing of Gr5a-, Gr43a-, Gr61a-, and Gr64f- but not Gr64a- or Gr64d-expressing neurons, significantly impaired L-arabinose memory (**Figure 3B**). Since silencing Gr5a- and Gr64f-expressing neurons also impairs L-arabinose consumption, the memory impairments may very well be due to an inability to detect L-arabinose (**Figure 3—figure supplement 1A**). Since silencing Gr61a neurons reduces detection, discrimination and memory, they may play a more general role in L- and D-arabinose detection and subsequent processing. Interestingly, silencing of Gr43a neurons had no effect on L-arabinose detection (**Figure 3—figure supplement 1A**) or D-arabinose preference (**Figure 3A**), but resulted in loss of L-arabinose memory (**Figure 3B**), suggesting Gr43a-expressing neurons play an important role in L-arabinose memory.

Gr43a is required for L-arabinose memory

Single gustatory neurons express multiple gustatory receptors. To determine which receptor within Gr43a neurons—Gr43a or some other receptor expressed by these neurons—is important for L-arabinose memory we trained receptor mutants with L-arabinose. Δ Gr43a, Δ Gr61a, and Δ Gr43a-61a flies all showed a significant reduction ($p < 0.01$) in long-term memory at 24 hr (**Figure 3C**). However, D-arabinose preference is maintained in the absence of any single known sugar receptor (**Figure 3D**). To determine whether L-arabinose memory phenotypes were simply due to detection deficits, we tested the mutants' ability to detect L-arabinose. Deletion of Gr43a had a small effect on L-arabinose detection, and deletion of Gr61a resulted in ~40% reduction (**Figure 3—figure supplement 2A**). D-arabinose detection was not altered by any single receptor deletion (**Figure 3—figure supplement 2B**). Taken together, these results suggest that Gr43a and Gr43a-expressing neurons are important to form long-term memory of L-arabinose, while Gr61a and Gr61a-expressing

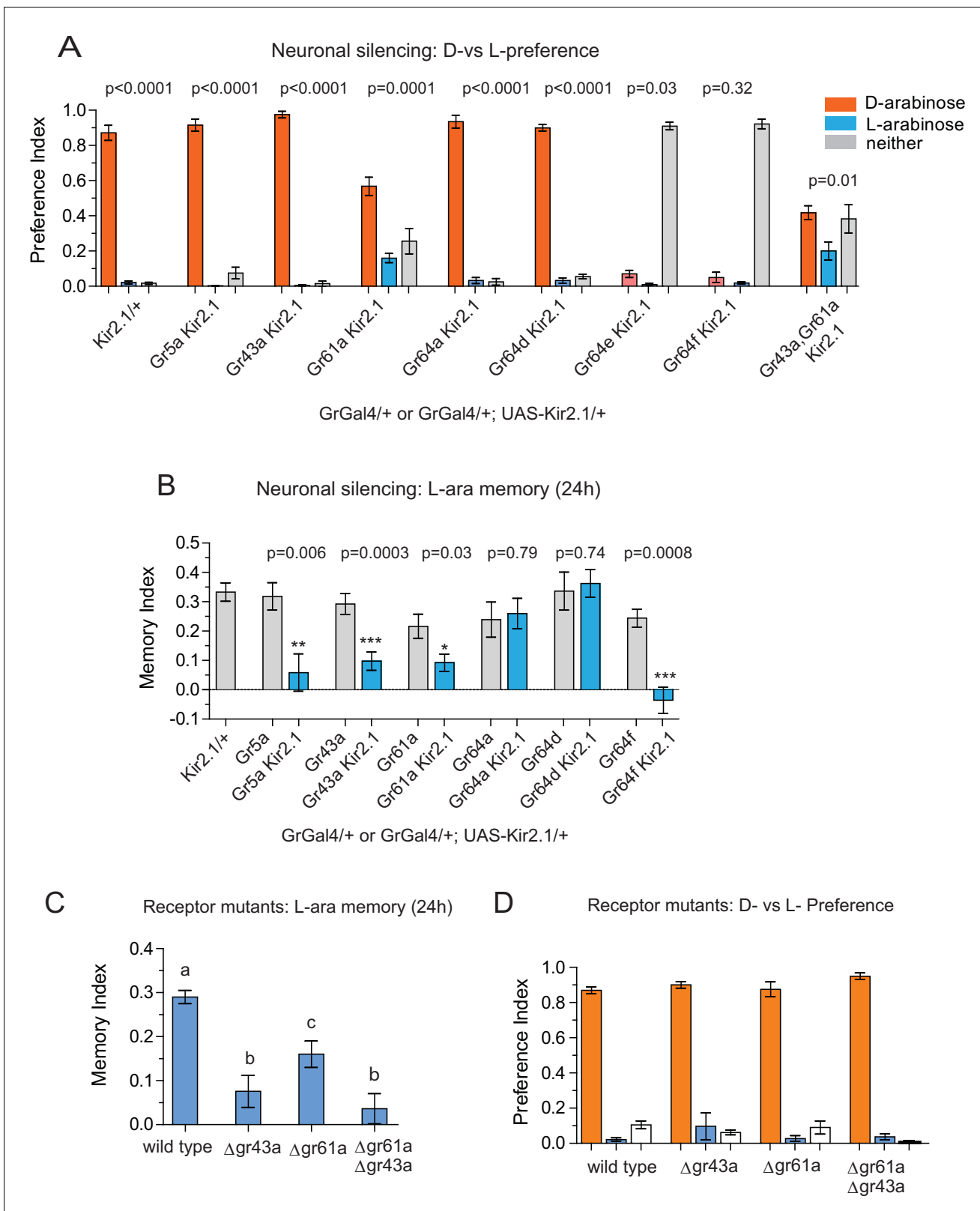


Figure 3. Gr43a- and Gr61a-expressing neurons are involved in D vs L preference and in L-arabinose memory. (A) Silencing of Gr61a-expressing neurons with Kir2.1 impaired D > L discrimination and preference; silencing Gr64e- and Gr64f-expressing neurons nearly eliminated detection of both sugars. (B) Silencing Gr43a- and Gr61a-expressing neurons impaired L-arabinose memory. Gr64aGAL4 and Gr64dGAL4, whose expression is restricted to LSO and VCISO neurons did not impair L-arabinose memory. Silencing Gr64f- and Gr5a-neurons reduce L-arabinose memory, but they also impair D vs L preference. (C) Receptor mutants Δ gr43a and Δ gr61a impair L-arabinose memory, but the double mutant Δ gr61a Δ gr43a does not. (D) Receptor mutants Δ gr43a and Δ gr61a do not impair D vs L preference, but the double mutant Δ gr61a Δ gr43a does. Error bars represent SEM. Statistical significance is indicated by asterisks (*p < 0.05, **p < 0.01, ***p < 0.001) or letters (a, b, c) indicating significant differences between groups. Figure 3 continued on next page

Figure 3 continued

L-arabinose detection. (see **Figure 3—figure supplement 1**). (C) Gr43a and Gr61a receptors are important for L-arabinose memory. (D) No single receptor mutant impaired D > L preference. For multiple samples, one-way ANOVA with Tukey's multiple comparisons test was performed, and significant differences ($p < 0.05$) are denoted by different letters. Results with error bars are means \pm s.e.m. ns, not significant. * ≤ 0.01 , ** ≤ 0.001 and *** ≤ 0.0001 .

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The following figure supplements are available for figure 3:

Figure supplement 1. Gr expressing neurons involved in D- and L-arabinose detection.

DOI: [10.7554/eLife.22283.011](https://doi.org/10.7554/eLife.22283.011)

Figure supplement 2. L- and D-arabinose detection do not rely on any single receptor.

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neurons are important for L- and D-arabinose detection and discrimination. These results, however, do not rule out the possibility that there may be an unidentified receptor that exclusively mediates D-arabinose preference or that L-arabinose memory uses other receptors in addition to Gr43a and Gr61a.

Peripheral Gr43a neurons are involved in L-arabinose memory

In *Drosophila*, gustatory receptors are present on the antennae, legs, wings, and labellae, and in the pharynx, gut, and central brain (Joseph and Carlson, 2015). The wide expression pattern, presence of multiple receptors in the same neurons, and different combinations of receptors in different neurons indicate that gustatory-receptor-expressing neurons in various locations may respond quite differently to different sugars (Thoma et al., 2016; Miyamoto and Amrein, 2014). We focused particularly on Gr43a-expressing neurons for their specific involvement in L-arabinose memory, and previous studies suggested they act as nutrient sensors (Miyamoto et al., 2012). We therefore sought to determine whether all Gr43a-expressing neurons or only a subset of Gr43a neurons are important for L-arabinose memory. As reported by others (Miyamoto et al., 2012; Park and Kwon, 2011), Gr43a^{GAL4} expression is consistently detected in four dorsolateral protocerebrum (DLP) neurons in the central brain, the LSO and VCSO neurons in the proboscis, two f5 neurons in the distal tarsi, and in the proventricular ganglion of the gut (Figure 4A). We selectively silenced the central brain DLP neurons using a Gr43aGAL4:ChaGAL80 (Miyamoto et al., 2012) combination (Figure 4B) or the LSO and VCSO neurons using Gr64aGAL4 and Gr64dGAL4 (Figure 3B). While silencing all Gr43a neurons impaired L-arabinose memory, silencing of just the DLP (Figure 4B), or just the LSO and VCSO neurons (Figure 3B) had no significant effect, suggesting that some combination of Gr43a-expressing neurons that includes the tarsal and/or gut neurons are the necessary Gr43a-expressing neurons for L-arabinose memory. Because silencing of Gr61a- and Gr5a-expressing neurons each blocked L-arabinose memory (Figure 3B) and neither Gr5a nor Gr61a expression can be detected in the gut, it seems that the tarsal Gr43a-expressing neurons are the important ones for L-arabinose memory. Previous studies suggested that in the f5 neurons in the distal tarsi, Gr43a is coexpressed with Gr61a (Figure 4—figure supplement 1A) (Freeman and Dahanukar, 2015). There were uncertainties about the coexpression of Gr43a and Gr5a in distal tarsi. However, split-GAL4 reconstitution assay suggests that Gr5a and Gr43a are likely to be coexpressed in one f5 neuron (likely f5V) in the distal tarsi (Figure 4—figure supplement 1B), in agreement with previous work (Miyamoto et al., 2012). Taken together, these results suggest that f5 neurons in the distal tarsi coexpressing Gr43a and some combination of Gr5a and Gr61a are involved in L-arabinose memory. However, these results do not rule out the possibility that other Gr43a neurons or other Gr-expressing neurons are involved in L-arabinose memory.

L-arabinose and D-arabinose activate peripheral Gr43a neurons to different extent

To understand how D- and L-arabinose generate different behavioral responses, we analyzed electrophysiological responses of f5V sensilla in the distal tarsi, which host neurons expressing Gr43a. D-arabinose consistently generated significantly more spikes over a range of concentrations (Figure 5A and B). Differences in electrophysiological response also manifested in calcium levels measured by GCaMP6, a genetically encoded calcium indicator (Chen et al., 2013). In Gr43a-

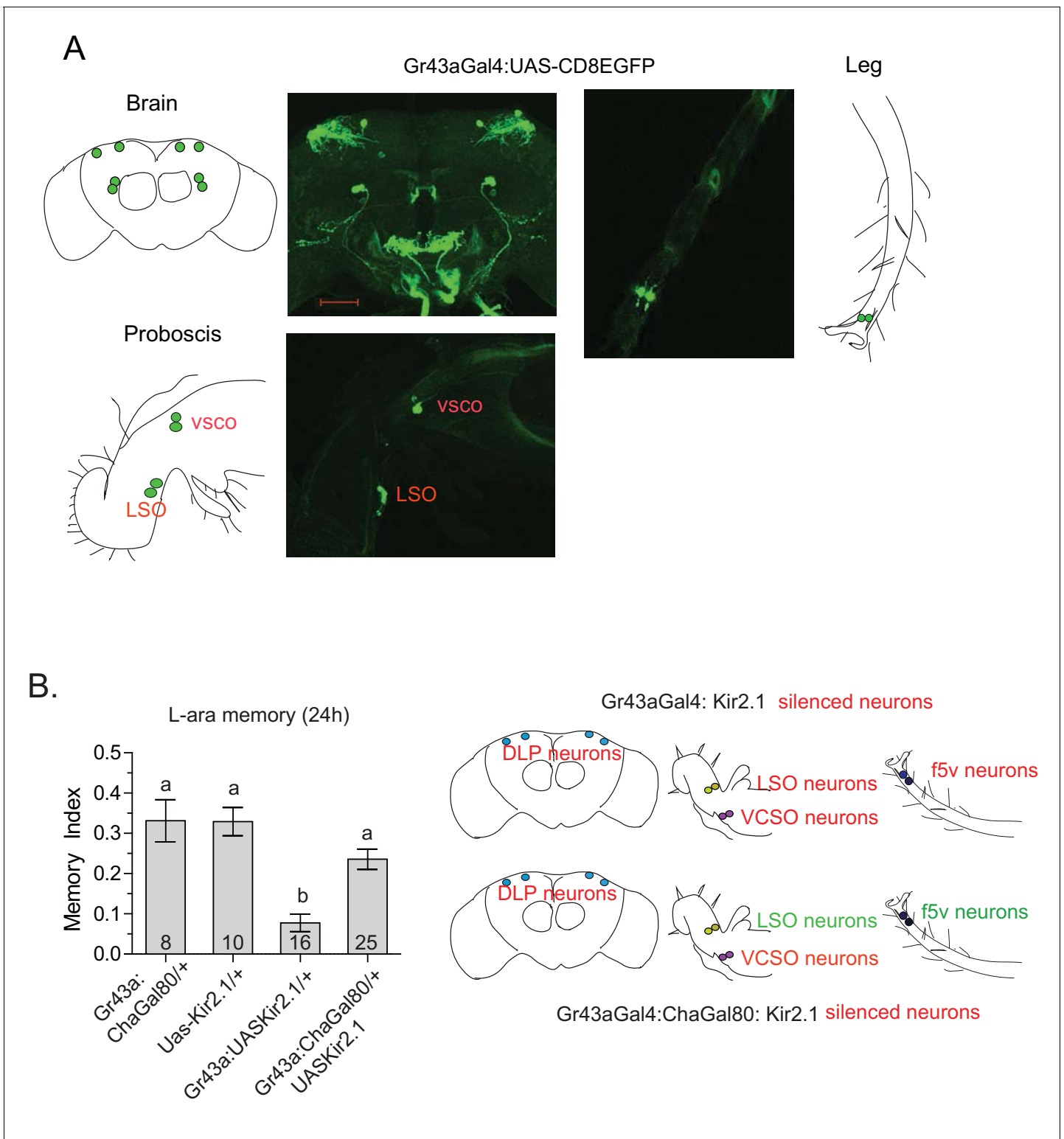


Figure 4. Tarsal Gr43a neurons are critical for L-arabinose memory. (A) Gr43a^{GAL4} neurons are observed in the dorsolateral protocerebrum, central brain, proboscis, leg, and gut (not shown). (B) Silencing only the dorsal protocerebral (DLP) and VCSO neurons does not impair L-arabinose memory. Left panel: memory score in various genetic backgrounds. Right panel, top: in Gr43a^{GAL4}/+; Kir2.1/+ flies, all indicated neurons are silenced (including proventricular neurons, not pictured). Bottom: in Gr43a^{GAL4}:ChaGal80/+; UAS-Kir2.1/+ flies, only the neurons indicated in red type are silenced. For multiple samples, one-way ANOVA with Tukey's multiple comparisons test was performed, and significant differences (p<0.05) are denoted by different letters. Results with error bars are means ± s.e.m. ns, not significant. *≤0.01, **≤0.001 and ***≤0.0001.

Figure 4 continued on next page

Figure 4 continued

DOI: 10.7554/eLife.22283.013

The following figure supplement is available for figure 4:

Figure supplement 1. Expression patterns of Gr-GAL4s.

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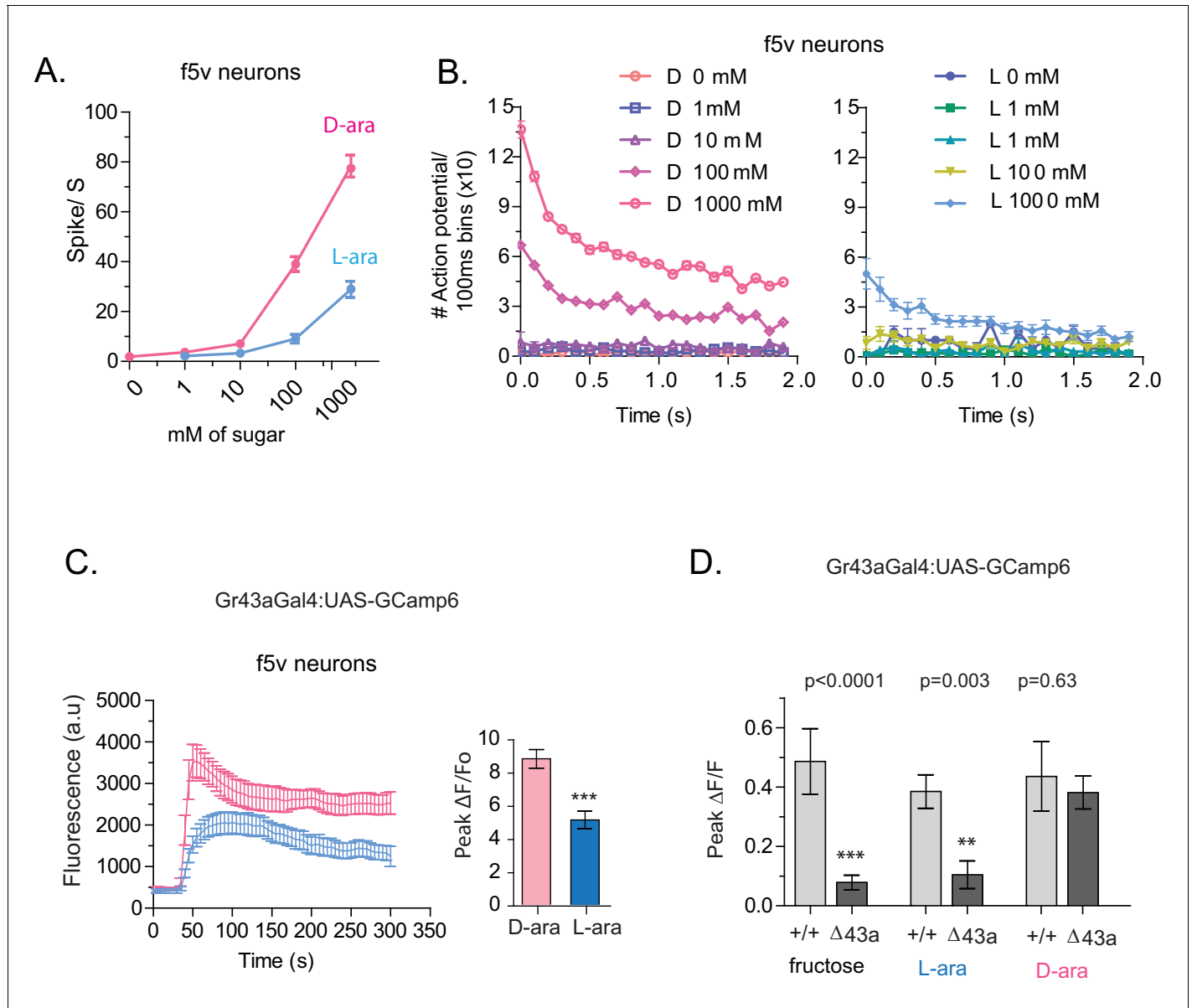


Figure 5. Tarsal Gr43a neurons respond differentially to D- and L-arabinose. (A) Spikes per second of recorded f5V tarsal neuron in response to D- or L-arabinose at various concentrations. (B) Spikes per second binned by 100 ms over the first two seconds of response. (C) The evoked-calcium activity of Gr43a^{GAL4} neurons in the distal tarsi. (D) Removal of Gr43a receptor impairs fructose and L-arabinose activation of Gr43a^{GAL4} neurons. Results with error bars are means ± s.e.m. ns, not significant. *≤0.01, **≤0.001 and ***≤0.0001.

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The following figure supplement is available for figure 5:

Figure supplement 1. Evoked-calcium activity of Gr43a neurons in response to D- and L-arabinose.

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expressing f5 neurons in distal tarsi, the peak calcium level was higher and reached more rapidly for D-arabinose than L-arabinose (**Figure 5C**). Removal of the Gr43a receptor from these neurons significantly reduced response to L-arabinose but not D-arabinose (**Figure 5D**), consistent with the idea that D-arabinose activates multiple receptors. In the proboscis LSO neurons, there was a quicker rise and fall in response to L-arabinose with a slower but more sustained activation in response to D-arabinose (**Figure 5—figure supplement 1A**). However, such differences between D- and L-arabinose-provoked responses are not universal: the average D- and L-arabinose responses of the central brain DLP neurons were similar in both magnitude and shape (**Figure 5—figure supplement 1B**). These results indicate that D- and L-arabinose can activate the same gustatory neurons to different extents and that differential activation depends on properties specific to each neuron.

Activation of Gr43a neurons can substitute the sugar reward to form associative memory

Because Gr43a^{GAL4} offered the most restricted set of neurons that was critical for L-arabinose memory, we sought to determine whether they also represented the minimum set of gustatory neurons sufficient for appetitive long-term memory formation. To this end, we asked whether activating Gr43a^{GAL4} neurons in the memory paradigm—in the absence of sugar—could generate an associative-appetitive memory (**Figure 6A**). dTrpA1, a temperature-sensitive cation channel, causes continuous activation of neurons at temperatures above 26°C (*Hamada et al., 2008*). Activation of Gr43a^{GAL4} neurons by dTrpA1, however, failed to substitute for the sugar reward (**Figure 6B**), although similar activation of a subset of dopaminergic neurons (R58E02-GAL4/+; dTrpA1/+) produced long-term appetitive memory as reported by others (*Liu et al., 2012*) (**Figure 6—figure supplement 1A**). These results suggested either that activation of Gr43a^{GAL4} neurons is necessary but not sufficient for L-arabinose memory, or that dTrpA1 does not approximate the activation required to produce long-term memory. Consistent with the latter possibility, activation of Gr43a neurons with the red-shifted channelrhodopsin variant ReaChR, a light-gated cation channel that depolarizes neurons in response to red light (*Lin et al., 2013*) produced associative memory: when flies expressing ReaChR in Gr43a^{GAL4} neurons were exposed to one odor without the light, and a second odor in the presence of red light, the flies subsequently preferred the light-associated odor (**Figure 6C**). Intriguingly, activation by the same amount of light evenly distributed was not effective in producing long-term memory, suggesting that these patterns evoked different levels or patterns of activity in Gr43a neurons; the nature of this activation is unknown at this time (**Figure 6C**). Finally, starvation is an important regulator of memory strength in the associative-appetitive paradigm—the hungrier the flies are, the better memories they form (*Krashes et al., 2009; Colomb et al., 2009*). Starvation also influenced the memory strength following Gr43a-neuron activation: the same pulsed light activation produced memory in starved but not fed flies (**Figure 6D**).

Since the f5 neurons in the distal tarsi express Gr5a in addition to Gr43a, we also activated Gr5a-expressing neurons. Similar to Gr43a neurons, activation of Gr5a-expressing neurons resulted in robust long-term memory (**Figure 6E**). Activation of Gr64a-expressing neurons, which labels the LSO and VCSO neurons, did not produce significant long-term memory (**Figure 6E**). Interestingly, Gr61a-expressing neurons are necessary but not sufficient to generate associative memory (**Figure 6E**), suggesting that activation of some Gr43a and Gr5a expressing neurons could be critical for memory processes, or that activation of the additional Gr61a-expressing neurons somehow weakens the co-expressing neurons' likelihood of generating memory. Taken together, these results suggest that activation of a subset of Gr43a-expressing neurons is sufficient to generate long-lasting associative memory. These observations further suggest that activation of the same neurons by different methods, perhaps leading to different activity levels/patterns, give rise to substantially different behavioral outcomes, consistent with other reports (*Clark et al., 2013; Seeger-Armbruster et al., 2015*). However, further work is necessary to determine exactly which subsets of neurons contribute to L-arabinose memory, and whether these neurons need to be activated in a specific pattern to elicit long-term memory.

Discussion

The observation that two similar sugars generate strikingly different behavioral responses can perhaps be best understood using the framework of 'incentive salience' in rewards, formulated by

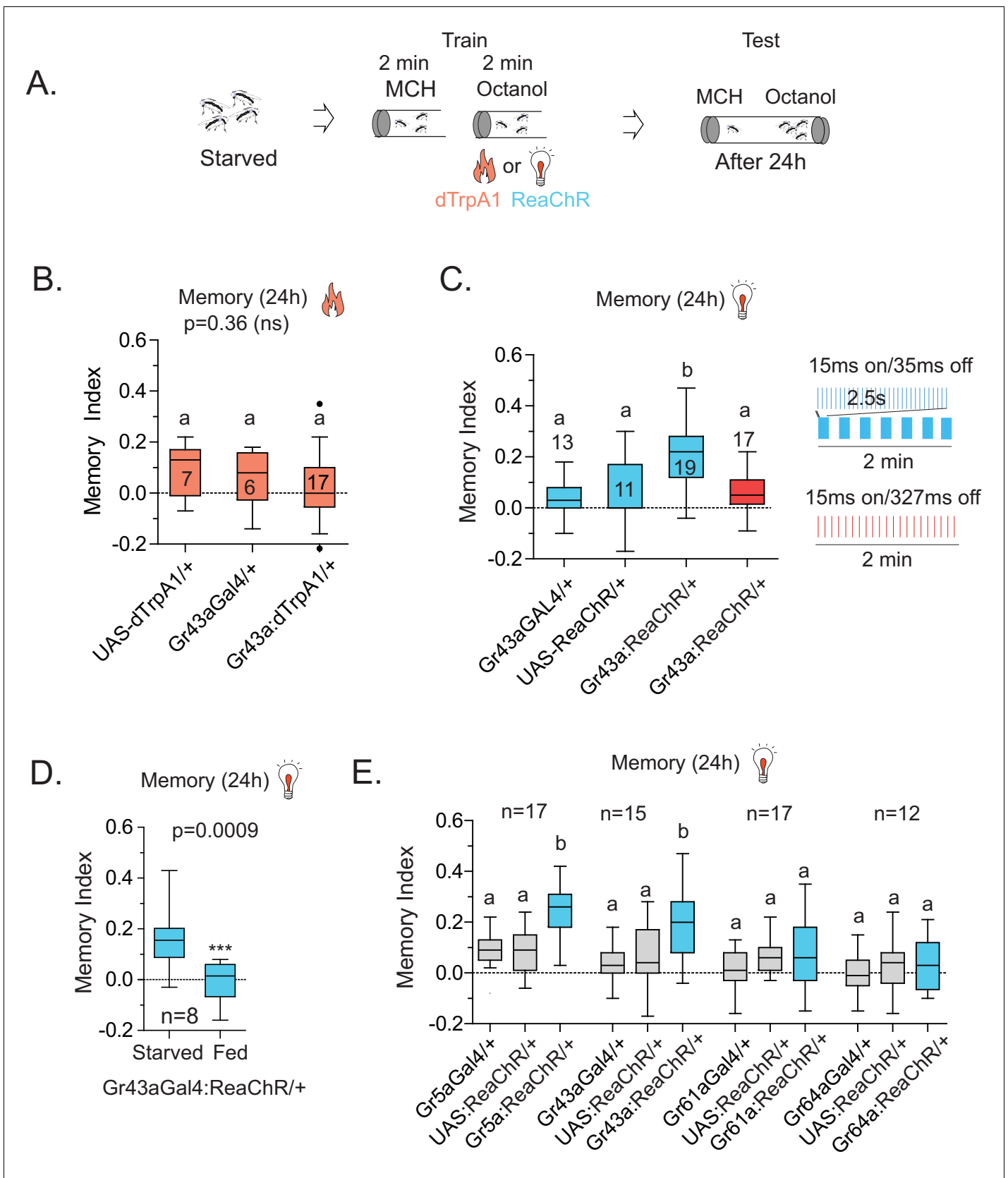


Figure 6. Activation of Gr43a neurons is sufficient to form rewarding associative memory. (A) Schematic of heat and light-activated associative olfactory training. (B) Activation of Gr43aGAL4 neurons by dTrpA1 (at 31 °C) does not induce long-term memory. (C) A 20 Hz, 15 ms pulse-width activation for 2.5 s Figure 6 continued on next page

Figure 6 continued

s, repeated every 20 s, induces long-term memory in flies expressing ReaChR in Gr43a^{GAL4} neurons; genetic controls do not show significant memory, and the same amount of light using the same pulse-width but distributed uniformly over the 2 min generates no memory (red). Schematics of light patterns are not to scale. (D) Optogenetic activation of Gr43a^{GAL4} neurons induces memory only in hungry flies, not in flies fed ad libitum. (E) Optogenetic activation of Gr43a- and Gr5a-expressing neurons leads to substantial 24 hr memory; activation of Gr61a- or Gr64a-expressing neurons does not. For multiple samples, one-way ANOVA with Tukey's multiple comparisons test was performed, and significant differences ($p < 0.05$) are denoted by different letters. Results with error bars are means \pm s.e.m. ns, not significant. * ≤ 0.01 , ** ≤ 0.001 and *** ≤ 0.0001 .

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The following figure supplement is available for figure 6:

Figure supplement 1. dTrpA1 activation of R58E02 neurons does not produce long-term memory.

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Berridge and Robinson (*Berridge and Robinson, 2003*), who divided reward percepts into 'liking' (conscious pleasure, hedonic) and 'wanting' (incentive salience). According to Berridge and Robinson, 'wanting' (incentive salience) is a component of rewards that transforms mere sensory information about rewards and their cues into 'attractive, desired, riveting incentives' and 'emerged early in evolution as an elementary form of stimulus-guided goal direction, to mediate pursuit of a few innate food or sex unconditioned stimuli' (*Berridge and Robinson, 2003*). In most cases, rewards that are 'liked' are usually also 'wanted', and in conventional formulations, they are considered effectively identical. But work on addiction and monetary reward on human suggest that 'wanting' and 'liking' are in fact dissociable, and while, in many cases, a behavioral response to an experience can predict the likelihood of memory formation, people can be motivated by cues remaining outside conscious awareness (*Pessiglione et al., 2007; Wise, 2002*). Here, we report that a similar distinction in reward perception may also exist in *Drosophila*, which is suggested by others (*Perry and Barron, 2013*): D-arabinose appears to preferentially involve the 'liking' component of the reward percept and L-arabinose the 'wanting'. For *Drosophila*, the incentive to remember L-arabinose is perhaps owing to the fact that it can inform a specific attribute of food, such as the ripening status of a fruit. Moreover, work in humans suggests that although 'liking' and 'wanting' both represent a positive reward, they utilize distinct neural processing (*Wise, 2002; Schultz, 2006*). Our observations with D- and L-arabinose now provide an opportunity to explore the neural basis of 'liking' and 'wanting', and how these reward percepts strengthen memory in the accessible nervous system of *Drosophila*.

Attributes of sugars important for long-term memory

The caloric value of a sugar has been found to be an important determinant of long-term appetitive memory (*Burke and Waddell, 2011; Fujita and Tanimura, 2011; Musso et al., 2015*), implying that flies quickly metabolize the sugar and that caloric evaluation somehow provides cues necessary to elicit long-term memory. We find that sugar with no caloric value can also produce long-term appetitive memories. One obvious possibility is that memories of sweet nutritious sugars are distinct from memories of sweet non-nutritious sugars. However, this seems so far not to be the case: a subset of higher order dopaminergic neurons (R58E02^{GAL4}) necessary for long-term memory of nutritious sucrose (*Liu et al., 2012*) is also required for non-nutritious L-arabinose (*Figure 6—figure supplement 1B*). Similarly, addition of sorbitol, a tasteless but nutritious sugar, enhances the memory of non-nutritious sugars like xylose and D-arabinose, but does not enhance the memory of nutritious sugars (*Burke and Waddell, 2011*). Adding sorbitol to L-arabinose had no additive effect on long-term memory (*Figure 6—figure supplement 1C*). It therefore appears that L-arabinose memory uses at least some of the same downstream neural circuitry as memory of nutritious sugars.

Whether memory of L-arabinose, a non-nutritious sugar, is an exception or represents a more general phenomenon is unclear since we have tested only a limited number of sugars in a particular behavioral paradigm. However, in addition to L-arabinose, L-fucose can also produce memory (*Figure 1E*); both are components of the pectin in many fruits' cell walls (*Dick and Labavitch, 1989; Ahmed and Labavitch, 1980*). It is therefore possible that these sugars may signal some specific attributes of ripening fruit—ripening is accompanied by breakdown of the fruit's cell walls—although neither of these sugars are present in fruits near the concentrations (1 M) used in memory assays. Nonetheless, these observations suggest that flies can quickly assess salient features of sugars—a sort of leading indicator of nutritional value—without the sugar's metabolic breakdown. This

approach to memory formation may allow flies to quickly recognize and remember potential foods using specific cues, a time advantage that could be vital in natural contexts.

Do insects distinguish structurally similar sugars? The taste modality of insects, particularly *Drosophila*, is reported to have limited discriminatory power and be primarily based on the intensity of the stimuli as opposed to the chemical nature of the sugar (Masek and Scott, 2010). Indeed we find that, apart from flies' differential preference for various sugars at equal concentrations, for immediate and short-term behavior this is largely true. However, we did not observe any obvious correlation between immediate behavior and long-term memories: flies immediate preference is L-fucose > D-arabinose > L-arabinose > L-sorbose (Figure 1D); for short-term memory, L-sorbose = D-arabinose \geq L-arabinose = L-fucose (Figure 1E); but in order of long-term memory score, L-arabinose \geq L-fucose \geq D-arabinose = L-sorbose (Figure 1F). These results indicate that while short-term responses are guided by palatability, long-term behavioral responses are guided by additional attributes of the sugars. It is not yet clear why D-arabinose is a less effective stimulus. Since D- and L-arabinose are both sweet, they may generate positive sensations in a different manner, or perhaps D-arabinose carries a negative value that over time reduces the positive association formed initially (or dampens the behavioral output).

Role of gustatory receptors in long-term appetitive memory

The gustatory receptors Gr5a, Gr43a, Gr61a, and Gr64a-f have been implicated in sugar detection (Fuji et al., 2015; Freeman and Dahanukar, 2015; Dahanukar et al., 2007; Scott et al., 2001; Dunipace et al., 2001; Montell, 2009; Jiao et al., 2007, 2008; Joseph and Carlson, 2015). Although exactly which Gr receptors are responsible for detecting which sugar remains somewhat controversial, two features of sweet-sensing gustatory receptors are generally agreed upon: first, different gustatory neurons express a number of Gr receptors in unique combinations; second, more than one receptor is typically involved in detecting a sugar (Fuji et al., 2015). However, the physiological consequences of this combinatorial expression of semi-redundant gustatory receptors remain uncertain. This study raises the possibility that gustatory neurons in different locations, expressing unique combinations of receptors, are responsible for discriminating chemically similar sugars and eliciting different behavioral responses. Consistent with this idea, previous studies suggested that Gr43a neurons in the central brain monitor hemolymph fructose levels and modulate feeding behavior (Miyamoto et al., 2012), while we find that these neurons are dispensable for L-arabinose memory, and that peripheral Gr43a-neurons are likely sufficient to signal the presence of a rewarding sugar and generate associative memories. These differences likely arise from the locations of these neurons, differentially expressed receptors, the presence or absence of various co-receptors, and the second-order neurons to which these neurons project. Exactly which or how many Gr43a-, Gr61a-, and Gr5a-expressing neurons in the periphery are sufficient for L-arabinose memory is currently unclear.

We also find that activation of Gr43a-expressing neurons by ReaChR but not dTrpA1 is able to generate appetitive memory, while artificially activating a subset of dopaminergic neurons (R58E02GAL4) by heat (dTrpA1) or light (ReaChR) both led to long-term memory (Figure 6—figure supplement 1A). How a difference in activity at the sensory level is conveyed to higher-order neurons, and how that difference is interpreted by the higher-order neurons, remains unclear. More concretely, why is dTrpA1 activation of a subset of dopamine neurons sufficient to generate memory, but dTrpA1 activation of Gr43a-expressing neurons is not? One possibility is that the activity requirements of neuromodulatory systems are less stringent than those for sensory coding, and that temporal selectivity occurs before the signal reaches these dopamine neurons. Alternatively, recent studies have indicated that dopaminergic neurons are functionally diverse, and that distinct population of dopaminergic neurons are involved in appetitive associative memory (Cohn et al., 2015; Huetteroth et al., 2015; Krashes et al., 2009; Berry et al., 2012; Aso et al., 2014; Yamagata et al., 2015; Berry et al., 2015; Musso et al., 2015; Schwaerzel et al., 2003). These reports raise the possibility that differing sensory inputs could activate different subsets of dopaminergic neurons.

How can structurally similar sugars generate differential activation? It is likely that although these sugars bind to some of the same receptors, the relative affinity of the receptors vary. In this regard, the fly sweet taste system may be similar to that of the mammalian system, where a single heteromeric receptor (T1R2 and T1R3) is responsible for detecting a large number of sweet substances,

with multiple discrete ligand-binding sites in each receptor responsible for generating diverse responses (Yarmolinsky *et al.*, 2009). We suspect that the differential engagement of multiple gustatory receptors leads similar chemicals to generate differential activation of the same neurons, and that differential activation and different ensembles of activated neurons allows higher-order neurons to decode the relevant features of sugars. We speculate that, at least in *Drosophila*, evaluation of a sugar's long-term salience may be encoded in the activation pattern of subsets of gustatory neurons, which allows rapid evaluation and remembering of nutritious food in complex environments.

Materials and methods

Fly stocks

Flies were generously shared by Dr. John Carlson (Gr43aGAL4-9/CyO; Gr61aGAL4-9/CyO; Gr5a-GAL4, Gr64GAL4), Dr. Hubert Amrein (UAS-Gr43a; Gr43a^{GAL4}, with first coding exon replaced with GAL4, serving as Δ Gr43a and used in crosses for behavioral training), Dr. Anupama Dahanukar (Gr61a-null mutant, Gr64a-null mutants and Gr5a-null mutants), Dr. Toshihiro Kitamoto (UAS-Shibire^{ts}), and Dr. Paul Garrity (UAS-dTrpA1). The wild-type Canton-S flies were generously provided by Dr. Scott Waddell and Dr. Troy Zars. Other fly stocks were obtained from Bloomington Fly Stock Center (UAS-Kir2.1 RRID:BDSC_6595; UAS-GCaMP3 RRID:BDSC_32235; UAS-GCaMP6m RRID:BDSC_42748; UAS-ReaChR RRID:BDSC_53749; Gr64eGAL4 RRID:BDSC_57667; Gr64fGAL4 RRID:BDSC_57669; Gr64dGAL4 RRID:BDSC_57665; Δ Gr64d/e RRID:BDSC_23628; Δ Gr64f RRID:BDSC_27883).

Sugars

Sugars were obtained from the following sources: D-arabinose, Sigma, cat#A3131-25G, lot# SLBB3223V,100M1365V and Fisher Bioreagents, cat# BP250425, lot# 114986; L-arabinose, Sigma, cat# A3256-100G, lot# BCBB3602V,098K0164 and USB Corporation, cat# 11406, lot# 4131874; L-sorbose, Sigma, cat# 85541, lot# BCBD8834V; L-fucose, Sigma, cat# F2252, lot# SLBB1522V; L-rhamnose monohydrate, Sigma, cat# R3875, lot# BCBD8824V; D-sorbitol, Sigma, cat# S1876, lot# 017 K0092; sucralose, Sigma, cat# 69293, lot# BCBF8524V; and saccharin sodium salt hydrate, Sigma, cat# S1002, lot# BCBF4560V; arabinogalactan, Food Science of Vermont, item# 026664342010.

Two-choice feeding assay using dye

The two-choice tests were performed essentially as previously described (Weiss *et al.*, 2011): 1- to 2-day-old male flies were collected in groups of 50, allowed to recover for 3 days, and food-deprived for approximately 22 hr in plastic tubes (VWR) containing kimwipes wetted with 3 ml of water. 1% agarose (Sigma) was mixed into 1 M sugar solution along with red or green food dye (1%, McCormick), and 15 μ l drops were pipetted into 60-well minitrays (Thermo Scientific). A hole large enough to fit a funnel was melted into the lid, and the 50 flies were allowed to feed for 5 min in complete darkness, with tape covering the lid hole. At the end of 5 min, the color in their abdomens was assessed under a dissecting microscope, and flies were counted as eating a sugar if any dye was visible in their abdomens or thorax. Flies eating a mix of the two were scored half for L-arabinose, half for D-arabinose. Preference and detection indices were calculated as (number of flies eating sugar)/(total number of flies). To rule out the color bias in the cases of choice between two sugars, half the experiments had the colors reversed. The feeding assay was carried out for 5 min, instead of a period of hours, because in the context of our particular behavioral paradigm the choices made by flies over a longer time period are not relevant.

Radioactive feeding assay

Two-choice radioactivity experiments were performed as described above, with the addition of 1 μ L of 1:5 diluted cytidine 5'-triphosphate [α -³²P] (3000 Ci/mmol 10mCi/ml, 1mCi; PerkinElmer) into 1.5 ml 1 M sugar solutions without dye; again sugars were pipetted onto the 60-well microtiter plate. After the 5-min feeding, flies were immediately placed on dry ice blocks, and five flies chosen at random were placed in each scintillation vial (Denville Scientific), homogenized, covered with 5 ml LSC-

cocktail (ScintiSafe, Fisher Scientific), and counted by scintillation counter (LS6500; Beckman Coulter).

Video monitoring of feeding assay

Video monitoring of feeding flies was performed using webcams (C160; Logitech). Four colorless drops of 1% agarose and 1 M sugar solution were placed on an empty 35 mM Petri dish (Falcon), one in each quadrant; two were L-arabinose and two were D-arabinose. Video was recorded for 30 min; trials in which the flies never found the sugars were discarded from analysis. Once the fly encountered a sugar solution, the behavior for next 5 min were quantified. We also examined the preference for other sugars, including sweet versus non-sweet sugars, to ensure that the experimental conditions did not influence the flies' choices.

Antibiotic feeding

Antibiotic experiments were carried out by placing approximately fifty 1- to 3-day-old flies into plastic tubes with kimwipes and 3 ml of either 1 M sucrose or 1 M sucrose with 100 µg/ml kanamycin, 500 µg/ml ampicillin, and 50 µg/ml tetracycline, for 24 hr. The antibiotic concentrations were chosen based on previously published work (*Ridley et al., 2013; Brummel et al., 2004; Sultan and Baker, 2001*). Flies were subsequently transferred to tubes with either 3 ml water or 3 ml water with 100 µg/ml kanamycin, 500 µg/ml ampicillin, and 50 µg/ml tetracycline, for another 22 hr. They were then trained with 1M L-arabinose as described below.

Survival assay

Survival curves were generated by placing fifty 3–5 day old flies in plastic tubes with kimwipes soaked in 2.5 ml of 1 M sugar solution. For each sugar solution tested, ten individual tubes were tracked, thus $n = 10$ for each solution. The number of dead flies was counted at 12, 24, 36, 60, and 72 hr.

Appetitive-olfactory conditioning

Olfactory training was carried out largely as previously described (*Krashes and Waddell, 2011*): 1- to 3-day-old flies were made hungry by placing groups of 50–70 flies in plastic tubes with kimwipes and tap water (time of starvation was determined by mortality rate: approximately 20–24 hr for homozygous lines; 24–30 hr for heterozygous crosses). Forty-seven microliters of 4-methylcyclohexanol (MCH; Sigma) and 42 µl of 3-octanol (OCT; Alfa Aesar) were separately diluted into two bubble humidifiers (B and F Medical) each containing 50 ml of mineral oil (Fisher Scientific); bubble humidifiers were connected in parallel by ¼-inch clear PVC tubing (VWR). 8 cm x 10 cm rectangles of filter paper (410, VWR) were soaked in water or 1 M sugar solution, and allowed to dry until the paper was damp, then rolled to fit tightly into the training tubes. Groups of 50–70 flies were moved into the t-maze, then into the water tube for 2 min while MCH odor passed through, moved back to the holding chamber in the t-maze for 30 s, then moved to the sugar tube for 2 min while OCT odor was flowing through. The next group of flies was trained reciprocally, where OCT was paired with water and MCH with sugar. Unless otherwise specified, after training flies were fed for 4 hr and restarved until testing 24 hr after training. Flies were tested by being given a choice between OCT and MCH in tubes with no filter paper; test duration was 2 min. Short-term memory was assayed 2 min after training. Memory index = [(number of flies in reward odor – number of flies in unrewarded odor)/(total number of flies)]. A memory index was calculated for each of the two reciprocal trials and then averaged; this average constituted an n of 1. Sucrose was frequently used as a daily standard, thus the large numbers of sucrose trials. For experiments with two or more controls, the experimental line was trained in parallel with one of the controls, and then again trained in parallel with the other control—thus the large n for both ChaGAL80 and ReaChR experiments.

Split-GAL4 construct

The split-GAL4 vectors (*Pfeiffer et al., 2010*) were made using the pHD-ScarlessDsRed vector (*Gratz et al., 2014*), DGRC #1364. To construct the Gr43a-VP16 vector, the 5' homology arm was inserted into the AarI restriction site by Gibson assembly (GACTGAACCGTGTAGGGA . . . TCCCCGCTTCTGAATTACT), immediately followed by the VP16 sequence (ATGGATAAAGCGGAA

TTAATTCC . . . CTGGGCGGCGGCAAGTAA) (addgene #26268). The 3' homology arm was inserted into the Sapl site (AGTAGTGACTCGGA . . . GAAGACCATATACGTC). CRISPR oligos were designed using <http://tools.flycrispr.molbio.wisc.edu/targetFinder/> (target sequences: AGAAC TGGGACCTTACAAGT and TACCTACCGCACGGGAATTT). To construct the Gr5a-DBD vector, the 5' homology arm: ACTTCGTTTGGCGTTTC . . . TAGAGCTTGATACACA, followed immediately by the GAL4 DNA-binding domain sequence (ATGCTGGAGATCCGC . . . ACAGTTGACTGTATCGTAA). The 3' homology arm for the Gr5a-DBD vector: ATGATGCTTTTCTTCGC . . . TCAACGGCCGTGC TCCTCT. CRISPR target sequences for the Gr5a locus were TGATTCCACACACGGGCATT and CGCACATCCAGCACACTGT. CRISPR gRNAs were ligated into the pU6-BbsI-chiRNA (addgene #45946) and pU6.2-BbsI-chiRNA vectors (Graz et al., 2013). DNA was mixed at a ratio of pHd-dsRed 500 ng/ul to U6-gRNA 100 ng/ul, and injected by BestGene, Inc. at a final concentration of 250 ng/ul.

Optogenetic and TrpA1 stimulation

Optogenetic activation was performed using the same hardware as previously published (Inagaki et al., 2014), except that two rows of six LEDs each were aligned parallel to the tube, 2 cm away, at 90° angles to each other. To minimize behavioral artifacts caused by strong visual stimulation, the red (627 nm, 161 lm @ 700 mA) Rebel LEDs were chosen, and the stimulation protocol (pulse width, intervals, and duration) was controlled by Arduino board and Arduino computer language. For dTrpA1 experiments, the relevant training tube was preheated to 31°C, and during training was wrapped in a ReptiTherm Under Tank Heater (RH-4; Zoo Med Laboratories); the temperature was held constant (at 31°C) by an electric temperature control with probe placed in between the wrapped layers (A419; Johnson Controls). The heater temperature required to maintain an internal tube temperature of 30°C was determined empirically.

Statistical analysis and number of trials (n)

All statistical analyses were performed using Graphpad Prism 5. All the data met the assumption of homogeneity of variance, therefore unpaired two-tailed t-test or one-way analysis of variance (ANOVA) was performed with Tukey post-hoc test between pairs of samples. ANOVA tests for significance were performed a probability value of 0.05 and more stringent values are listed in each figure where applicable. For all experiments, each *n* is considered a biological replicate; separate trials used independent samples of genetically identical flies. For two-choice experiments, a single *n* constitutes a population measure generated from 50 male flies. The preference index indicates the proportion of flies eating the sugar, which was determined by scoring visible color in the abdomen or thorax. For video monitoring, each *n* constitutes a single fly. For survival curves, each *n* is a population measure generated by 50 flies placed in a tube with 1M sugar. Percent survival indicates the percentage of flies alive at each timepoint. For olfactory training experiments with sugar, heat, and light: one trial consists of giving a group of approximately 50–70 flies water and 3-octanol for 2 min, waiting 30 s, then giving sugar and 4-methylcyclohexanol for 2 min. Another group is trained with water and 4-methylcyclohexanol, then sugar and 3-octanol. Memory indices are calculated for each of these two trials and averaged. This average constitutes a single *n*, which is approximately 100–140 flies. Based on the previous and ongoing experimental effect sizes, 8–10 of these double trials were generally judged to be adequate for memory experiments, unless effect sizes were strikingly large or variable. The more dramatic effect sizes and smaller variability of preference assays allowed a smaller number of trials, generally 4. In all long-term memory experiments, experimental manipulations for which a negative result was plausible or expected were always trained alongside a positive control. This is the reason for conspicuously large numbers of trials with sucrose and L-arabinose compared to other sugars or manipulations. Similarly, for experimental groups needing to be compared to two or more controls, the experimental group was first trained alongside one of the control groups, and then again trained alongside the other control group (s). This is the reason for large numbers of trials in, for example, the ChaGAL80 and ReaChR experiments.

Immunostaining

Tissues were dissected in PBS (Sigma), and fixed in 4% paraformaldehyde (Electron Microscopy Sciences) in PBS-Triton. 3% (PBST) (Sigma) for 1–2 hr. They were washed in PBS-Triton: 3% five times

for 15 min each time, and blocked in PBST with 10% normal goat serum (Vector Laboratories) for 2 hr. Rabbit anti-GFP IgG (MBL International Corporation) was diluted 1:1000 in the blocking solution and centrifuged at 14,000 r/min for 10 min at 4°C. Tissues were incubated with primary antibody overnight at 4°C, then washed again with PBST for 15 min, five times. Anti-rabbit IgG Alexa Fluor 488 (Life Technologies, now ThermoFisher Scientific, Waltham, MA) was diluted 1:1000 in blocking solution, and incubated with the tissues overnight. Tissues were again washed five times, and mounted in Vectashield (Vector Laboratories) on slides with doubled clear reinforcement labels (Avery); No. 1 ½ coverslips were used (VWR). Images were acquired on a Zeiss Pascal confocal microscope with a Plan Apochromat 20 × 0.8 NA objective. GFP fluorescence was excited at 488 nm and emission was collected through a 505–530 nm bandpass filter.

Calcium imaging

Tissues from Gr43a^{GAL4} × UAS-GCaMP3 or UAS-GCaMP6med flies were prepared largely as described previously (Miyamoto *et al.*, 2012). Two- to 7-day-old flies were used. All tissues were dissected in Ringers solution (5 mM HEPES, 130 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂); legs were removed from the fly, placed on a 50 mm glass-bottomed dish No 1.5 (Mattek), and immobilized with a 1.5 μl drop of 2-hydroxyethylagarose (Sigma). After the agarose firmed, 20 μl of Ringers was added to cover the leg. In D- vs L-arabinose comparisons, both front legs of the fly were used as matched controls. Brains adhered to the dish without need for agarose when placed into a 30 μL bubble of Ringers. Proboscis imaging was performed with the proboscis upside down on the plate, so that the dorsal proboscis was contacting the dish; the proboscis was immobilized with 1.5 μl of agarose and again covered with 30 μl of Ringers solution. Only one sugar was tested per tissue sample. Images were collected at least 40 s before sugar was added; sugar was added at 2x concentration, in the same volume as the Ringers covering the tissues. Because the training paradigm uses high concentrations (up to 1 M) of sugar, we used 500 mM sugar concentrations for the leg and proboscis imaging. However, for the brain, 500 mM appeared to cause osmolarity-induced shrinking, so brain imaging used 100 mM sugars. Leg imaging was performed at approximately one stack per 5–7 s; proboscis imaging at approximately one stack per 13 s; brain imaging at approximately one per 14 s. Only tissues that showed a response were used in analysis, although tissues that didn't respond were checked for viability by adding fructose as a positive control. Images were acquired on a Zeiss Pascal confocal microscope with a Plan Apochromat 20 × 0.8 NA objective. GFP fluorescence was excited at 488 nm and emission was collected through a 505–530 nm bandpass filter. For calcium imaging of the leg with ΔGr43a-GAL4 × UAS-GCaMP3 and ΔGr43a-GAL4; UAS-GCaMP6med flies, images were acquired on an Ultraview Vox (PerkinElmer) with a Plan Apochromat 20 × 0.8 NA objective at approximately one stack per 10 s; GFP fluorescence was excited at 488 nm and collected through a 525–550 nm bandpass filter. Analysis was performed in ImageJ (NIH) using in-house plugins: z-stack images were sum-projected and camera background was subtracted by selecting a region of interest away from the tissue. Where needed, the StackReg registration plugin was used to minimize movement artifacts (Thévenaz *et al.*, 1998). Measurements were always taken by encircling cell bodies. In tissues with more than one neuron visible, the response of each neuron was analyzed separately and then averaged to generate an average response for that single tissue; this average constituted a single *n* and was used with others to generate average response curves and peak ΔF/F₀. Peak ΔF/F₀ measurements were made by taking the first peak value, and dividing by the average of five timepoints immediately preceding the rise. To generate normalized fluorescence curves, individual tissue averages were aligned by the first timepoint of the rise. Curves for leg and proboscis were linearly resampled at 3 s; brain at 5 s. Curves were then min/max normalized, and average trajectories were calculated. Error bars were calculated as standard error in the mean. Average curves were plotted in GraphPad Prism 5.

CAFÉ assay

One-day-old Canton S adult flies (males and females) were transferred to fresh standard food medium for 1 day and then starved (with free access to water) for 18–22 hr. These flies were then transferred by groups of 20 into plastic boxes (Sellier *et al.*, 2011). Each box had a row of five capillary tubes (5 μl minicaps, Hirschmann LaborGeräte, Germany), filled with a dilution of sugar mixed with a red dye (erythrosine 0.374 mg/ml; Sigma France). The concentrations of sugar (L- and

D-arabinose, Sigma, France) were: 1 M, 100 mM, 10 mM, 1 mM and 0 mM. Each box was monitored with a webcam (HD Pro C920 or QuickCam Pro 9000, Logitech). The boxes and cameras were housed in a climatic chamber maintaining a temperature of 25°C and 80% H.R. (DR-36 VL, CLF Plant Climatics GmbH, Germany). For each box, images were acquired at a rate of one image/ min for 2 hr using the software VisionGS, Germany. The stack of images was then transferred to ImageJ (*Abramoff et al., 2004*) and the liquid level of each capillary was analyzed using a Java plugin, and subsequently transferred to Excel. Results are expressed as the mean of the change of the liquid level in each capillary (D-arabinose: n = 12; L-arabinose: n = 10 boxes). Error bars are computed as the standard error to the mean (s.e.m.).

Electrophysiological recordings

Tip-recording was performed as previously described (*French et al., 2015*). Briefly, adult flies (3- to 4-day old) were anesthetized on ice and immobilized on a putty platform (UHU stick), using thin stripes of tape. They were then disposed under a stereomicroscope (MZ12, Leica) and specific sensilla from the proboscis or from the legs were stimulated and recorded, using a TasteProbe amplifier (DT-02, Syntech, Germany; *Marion-Poll and Pers, 1996*) connected to a general purpose amplifier (CyberAmp 320, Data Translation, USA) which further amplified (x100) and filtered the signal (10 Hz-2800 Hz). The stimulus electrode contained tricholine citrate (TCC 30 mM), in order to allow an electric contact to be established with the sensillum and to inhibit firing activity arising from water-sensitive cells (*Wieczorek and Wolff, 1989*). A reference electrode was connected to the abdomen of the fly, using a drop of electrocardiogram gel. Each stimulation lasted 2 s and was digitized at 10 kHz, 16 bits during 2 s (DT9818, Data Translation, USA). The data acquisition, spike detection and sorting was performed under a program, dbWave. The results were subsequently transferred to Excel, and expressed as the mean (n = 8–15 measures). Error bars were computed as the s.e.m.

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KS and JM conceived of the project, designed the experiments, analyzed the data, and prepared the manuscript. JM, HJ, AVA., and CPS performed the experiments. JU, BS, and JL assisted with imaging and image analysis. ZY built the optogenetic apparatus. KS and JM wrote the paper and made the figures. MAA performed the electrophysiological observations, Palak Rawat performed CAFE experiments, and FMP analyzed the CAFE and electrophysiology data. We are especially grateful to Dr. John Carlson of Yale University for reagents and suggestions in the early stages of the project. We thank Wanda Colon Cesario, Adrienne van Antwerp, and Abiel Trevino for assistance with sugar preference tests and calcium imaging. We thank Tony Torello for help in constructing the optogenetic apparatus. We thank Liying Li, Amitabha Majumdar, Gunther Hollopeter, Jay Unruh, and Brian Slaughter for important discussions. This work was supported by the Stowers Institute for Medical Research.

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Figures and figure supplements

Immediate perception of a reward is distinct from the reward's long-term salience

John P McGinnis *et al*

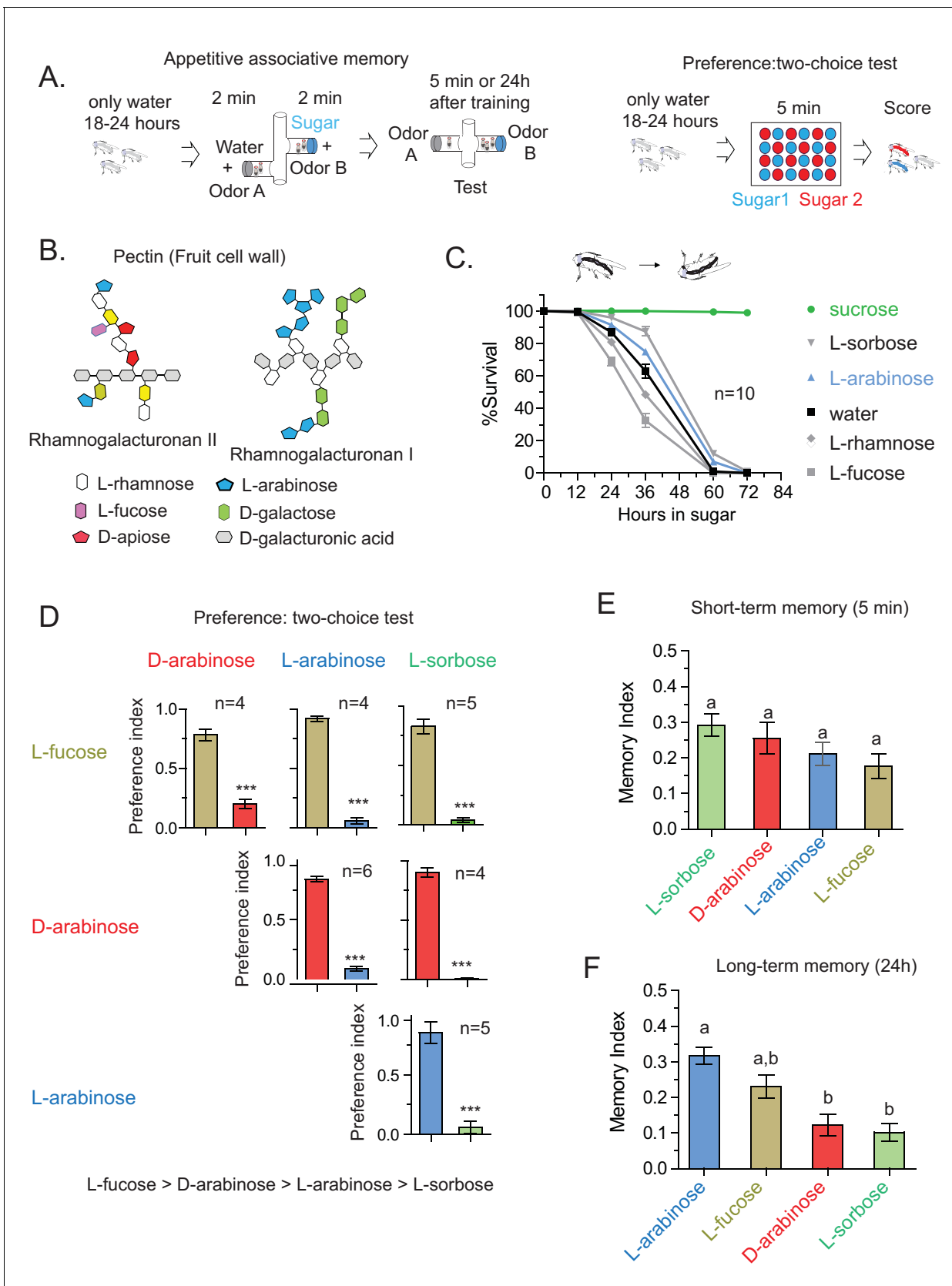


Figure 1. Flies' immediate preference for a sugar is not predictive of their long-term memory: various sugars from fruits. (A) Schematic of behavioral assays. In the appetitive associative memory paradigm, hungry flies are trained for 2 min with the sugar-odor pair and memory is assayed 5 min or 24h after training. In the preference test, flies are trained for 5 min with a two-choice test and their preference is scored. (B) Chemical structure of pectin, a major component of fruit cell walls, showing the various sugars it contains. (C) Survival curves for flies in different sugars. (D-F) Preference and memory indices for various sugars. Preference index is the proportion of flies that chose a particular sugar in a two-choice test. Memory index is the proportion of flies that chose a particular sugar in a two-choice test after 5 min (E) or 24h (F) of training. Error bars represent SEM. Significance levels: *** p < 0.001, a p < 0.05, b p < 0.01.

Figure 1 continued

by subsequently giving a choice between the two odors. In the preference assay, hungry flies are given a choice between two sugars mixed with different colors; after 5 min color of the abdomen is used to assess consumption. (B) Schematic of selected pectic polysaccharides present in fruits' cell walls, adapted from Harholt et al. (Harholt et al., 2010). (C) Survival percentages for flies given solely 1 M sugar solutions. $n = 10$ (50 flies per n) for each time point. (D) Two-choice tests comparing flies' preference for each of four sugars when both sugars are presented side-by-side for 5 min (50 flies per n). (E) Short-term (5 min) associative memory scores for the sugars. ($n = 7-11$) (F) Long-term (24 hr) associative memory scores for the sugars. L-fucose is a component of pectin as well, although the amount is low compared to L-arabinose. ($n = 20-24$) Memory scores labeled *a* are significantly different (<0.05) from bars labeled *b*, analyzed by one way ANOVA with Tukey's multiple comparisons test. Detailed explanations of what constitutes a single n is found in Materials and methods. Results with error bars are means \pm s.e.m.

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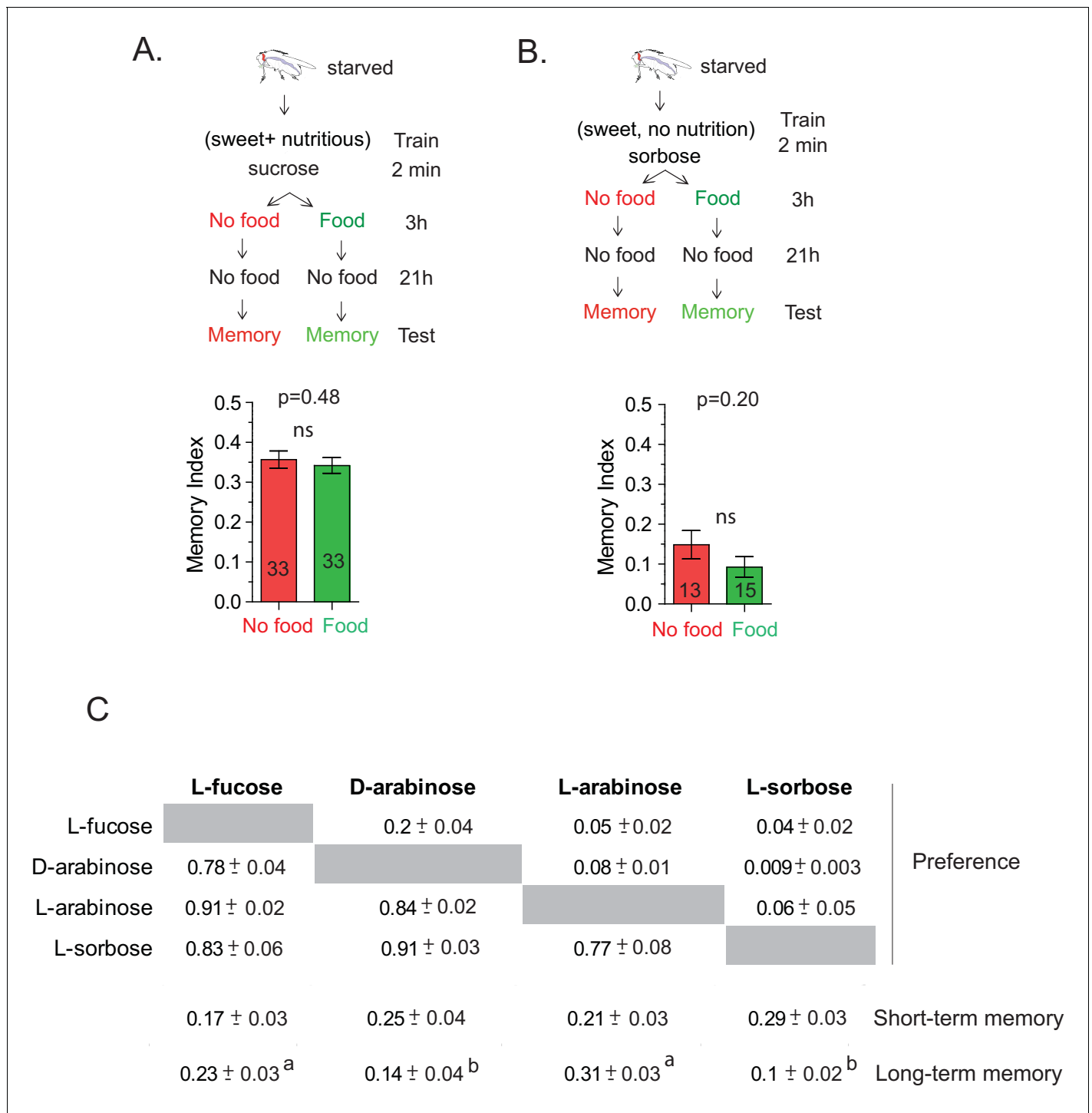


Figure 1—figure supplement 1. The CS-US association occurs during the two-minute training. (A) Flies trained with sucrose, which produces robust 24 hr memory, were immediately provided either rich nutritious food or water. If sucrose’s nutritional value is assessed beyond the 2-min training, nutritious food immediately after training may interfere with the fly’s ability to attribute its nutritional status to the 2-min training, and thus interfere with memory formation. However, flies trained with 1 M sucrose and given food or no food for 3 hr post-training showed similar memory, unpaired t-test, $p=0.480$. (B) Flies trained with L-sorbose—a sweet but non-nutritious sugar that produces short- but not long-term memory—were immediately fed sucrose. If the nutritional evaluation occurred after the 2-min training, immediate feeding on sucrose may substitute as a nutritional cue, resulting in enhanced long-term memory. However, flies trained with 1 M L-sorbose and given sucrose immediately after training showed similar memory to flies not given sucrose, suggesting that the critical association period was confined to the 2-min training, unpaired t-test, $p=0.207$. (C) Matrix of flies’ preference for one sugar (top) when paired side-by-side with another sugar (side) for 5 min. Each comparison was tested with four independent trials, *Figure 1—figure supplement 1 continued on next page*

Figure 1—figure supplement 1 continued

50 flies per trial. Numbers are the proportion eating the sugar listed at top. (Proportions may not sum to 1.0; often several flies would not eat either sugar.) Short-term memory and long-term memory averages shown below with \pm s.e.m. Memory scores labeled *a* are significantly different ($p < 0.05$) from bars labeled *b*, analyzed by one-way ANOVA with Tukey's multiple comparisons test.

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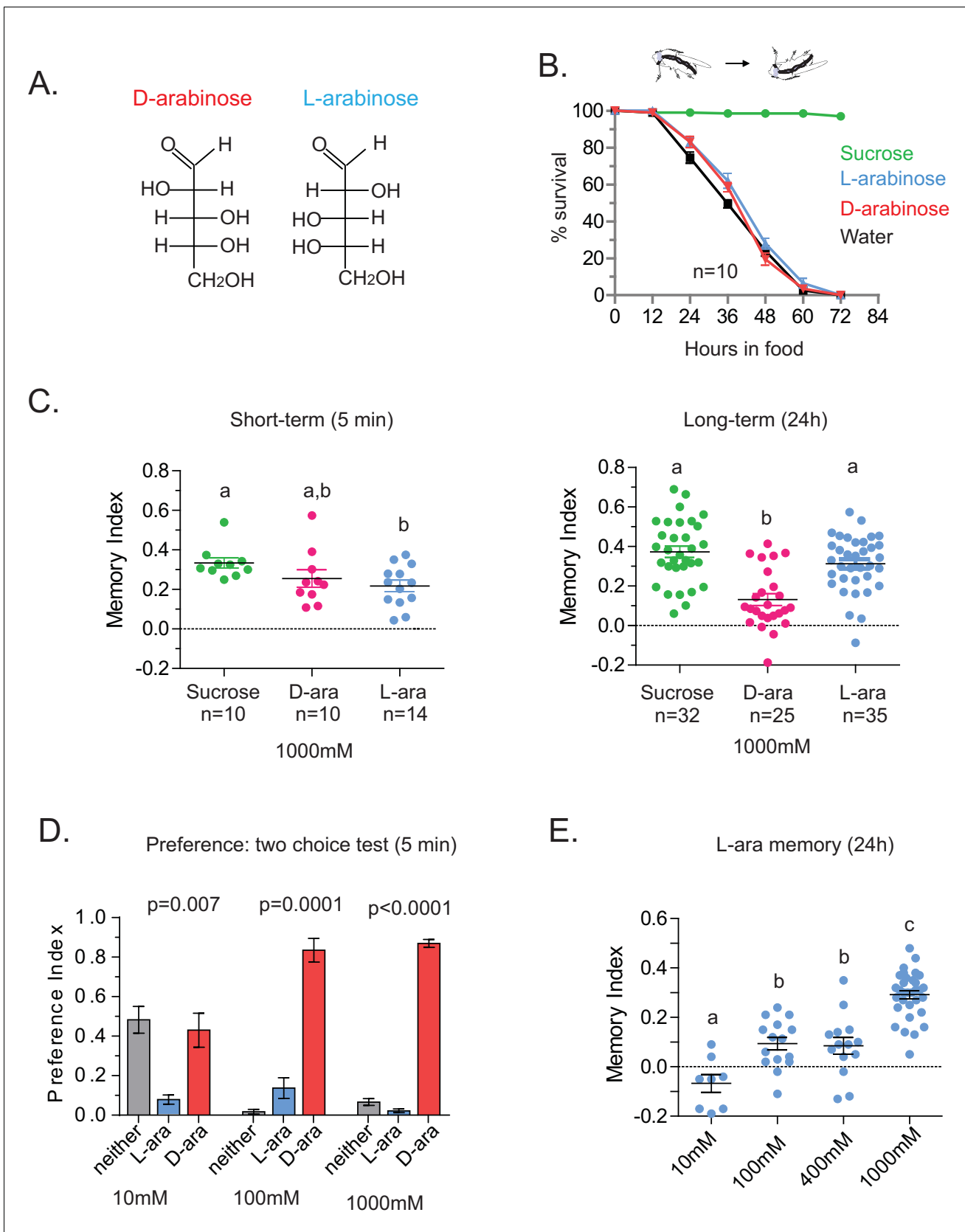


Figure 2. Flies' immediate preference for a sugar is not predictive of their long-term memory: L- vs D-arabinose. (A) Structures of D- and L-arabinose. (B) Survival percentages for flies given solely 1 M sugar solutions. $n = 10$ (50 flies per n) for each time point. (C) Short- and long-term memory of sucrose
 Figure 2 continued on next page

Figure 2 continued

and D- and L-arabinose. (D) Two-choice tests comparing flies' preference for D- and L-arabinose when both sugars are presented side-by-side for 5 mins. $n = 4$ (50 flies per n). (E) Long-term (24 hr) memory scores for increasing concentrations of L-arabinose. Results with error bars are means \pm s.e.m. ns, not significant. $^* \leq 0.01$, $^{**} \leq 0.001$ and $^{***} \leq 0.0001$. The significant differences ($p < 0.05$) between conditions in **Figure 2C and E** were analyzed by one-way ANOVA with Tukey's multiple comparisons test and differences are denoted by different letters. Detailed explanations of what constitutes a single n is found in Materials and methods.

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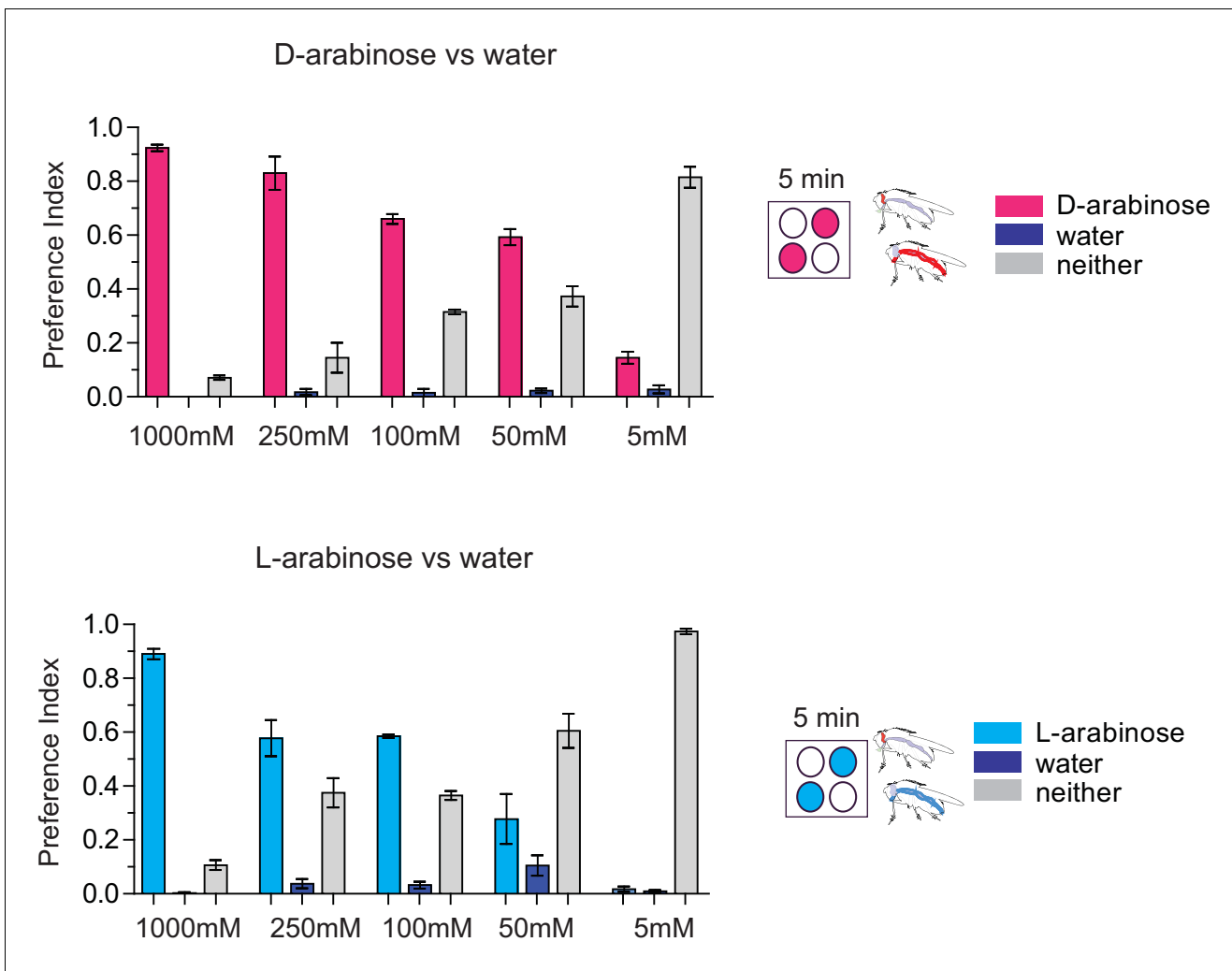


Figure 2—figure supplement 1. Palatability of D- and L-arabinose over a concentration range. Fifty male flies per trial were given water alone for 24–36 hr, then put on a microtiter plate checked with food-dye-labeled water and either D-arabinose or L-arabinose at various concentrations. After 5 min, flies were removed and the color visible in the abdomen was scored.

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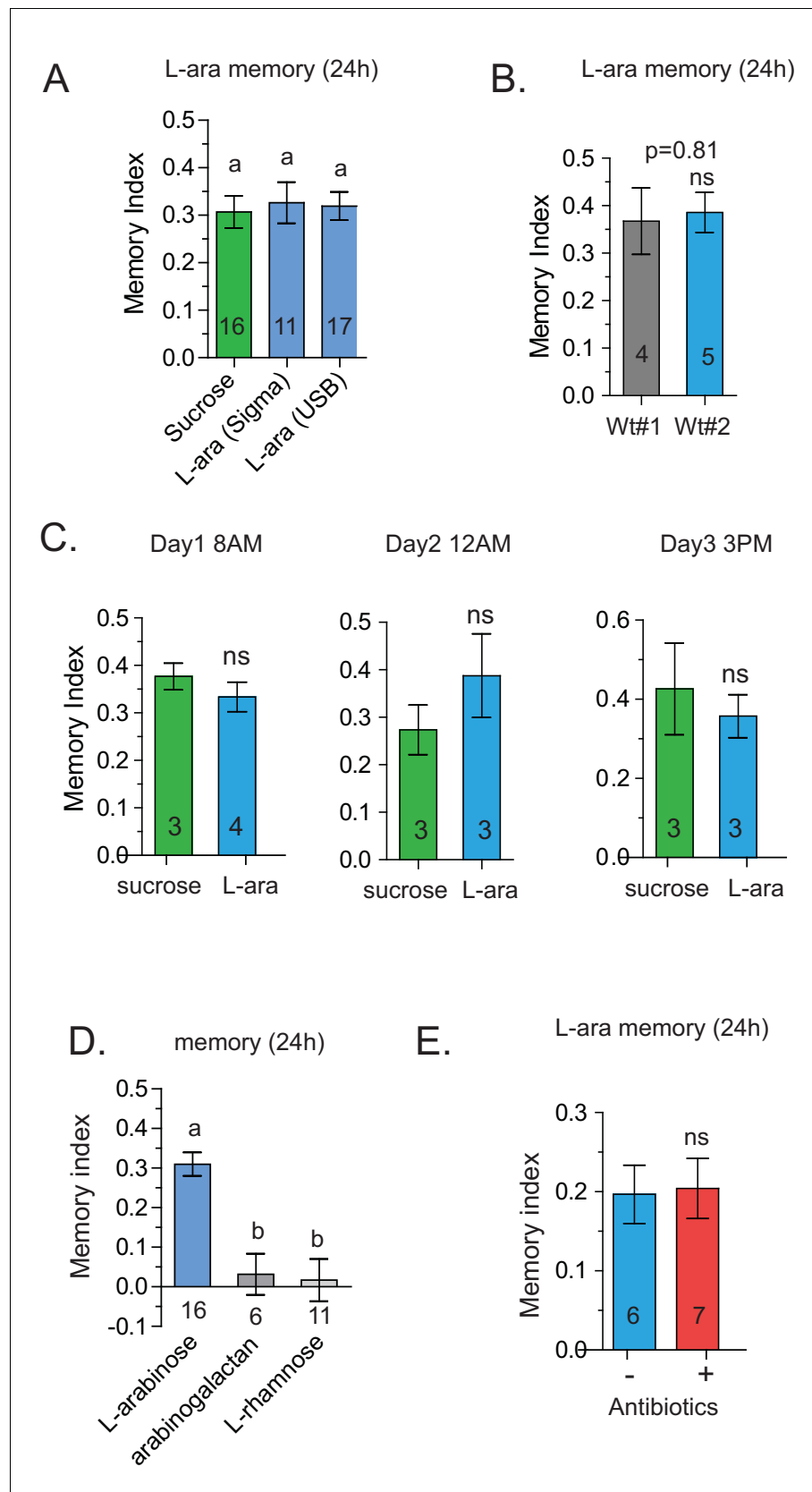


Figure 2—figure supplement 2. Specificity of L-arabinose memory. (A) L-arabinose from different sources (USB and Sigma) generated equivalent long-term memory, controlling for other contaminating sugars. (B) Canton-S flies
 Figure 2—figure supplement 2 continued on next page

Figure 2—figure supplement 2 continued

obtained from two different labs show similar long-term memory when trained with L-arabinose. (C) Since flies' feeding behavior can be influenced by the time of day, flies were tested at different times on consecutive days by different experimenters. Under all testing conditions, flies formed long-term memories of L-arabinose. (D) While L-arabinose forms memory, an L-arabinose-galactose polymer does not; neither does another natural L sugar, L-rhamnose, demonstrating the selectivity of L-arabinose memory. (E) Flies fed a cocktail of three broad-spectrum antibiotics (kanamycin, ampicillin, and tetracycline) for the 48 hr before training show memory indistinguishable from untreated control flies. For two samples unpaired two tailed t-tests and for multiple samples one-way ANOVA with Tukey's multiple comparisons test were performed, and significant differences ($p < 0.05$) are denoted by different letters. Results with error bars are means \pm s.e.m. ns, not significant. * ≤ 0.01 , ** ≤ 0.001 and *** ≤ 0.0001 . DOI: [10.7554/eLife.22283.007](https://doi.org/10.7554/eLife.22283.007)

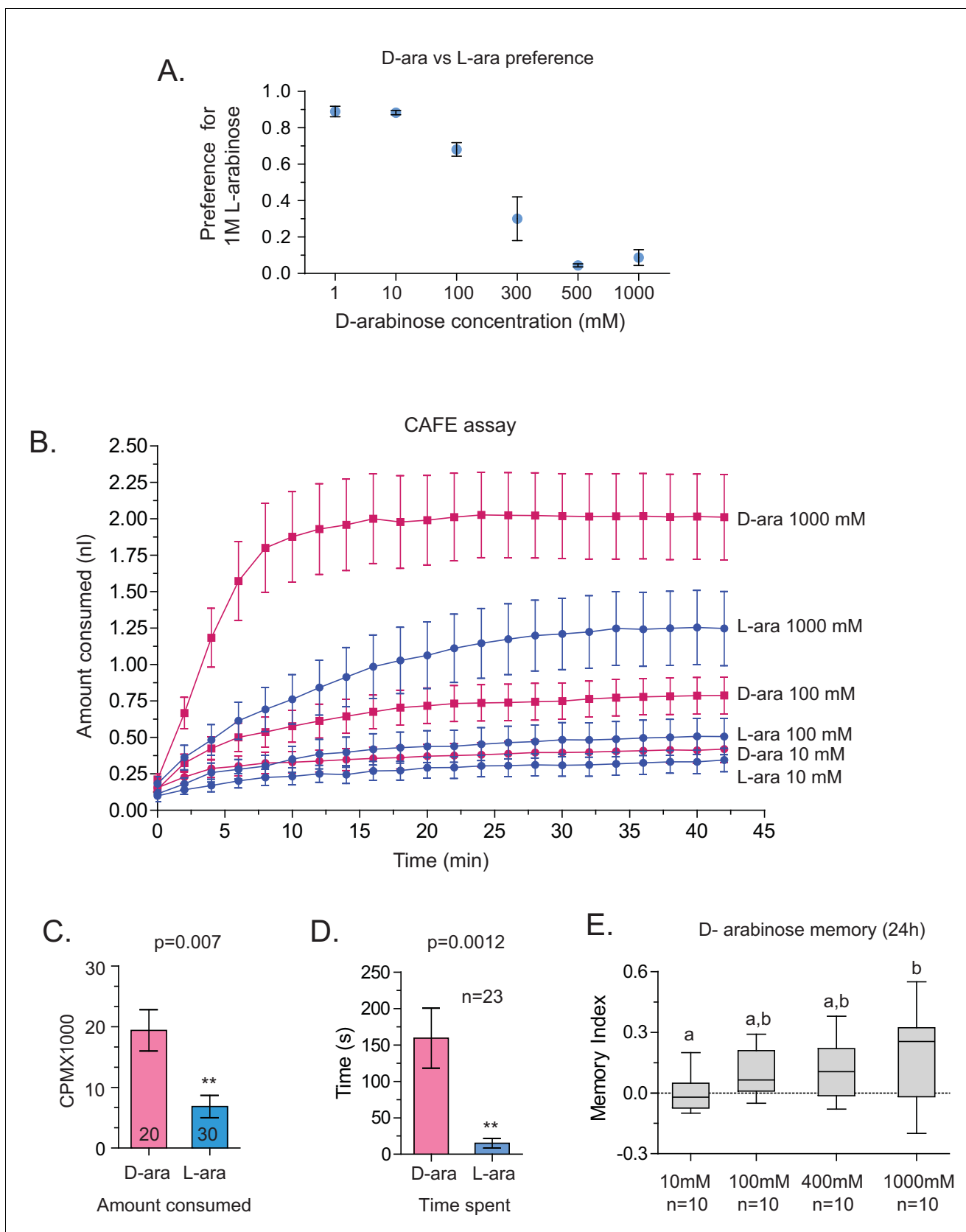


Figure 2—figure supplement 3. Although both are sweet, D-arabinose is preferred over L-arabinose. (A) At equal concentrations, flies overwhelmingly prefer D-arabinose to L-arabinose; the preference begins to shift when L-arabinose is ≥ 3 times more concentrated than D-arabinose. (B) CAFE assay
 Figure 2—figure supplement 3 continued on next page

Figure 2—figure supplement 3 continued

quantifying flies' intake of D- and L-arabinose across a range of concentrations. (C) In 5 min, flies consume more radioactive [32]P-mixed D-arabinose than radioactive [32]P-mixed L-arabinose during two-choice tests when either sugar is tested separately against water. (D) Single flies monitored by video spend more time on 1 M D-arabinose than 1 M L-arabinose when the two are presented side-by-side. (E) The greater the concentration of D-arabinose, the better the memory score. This suggests that the flies are not eating so much D-arabinose that they become sick, and would otherwise remember if not for eating large amounts of sugar. Memory does not improve at lower concentrations. For multiple samples, one way ANOVA with Tukey's multiple comparisons test was performed, and significant differences ($p < 0.05$) are denoted by different letters. Results with error bars are means \pm s.e.m. ns, not significant. * ≤ 0.01 , ** ≤ 0.001 and *** ≤ 0.0001 .

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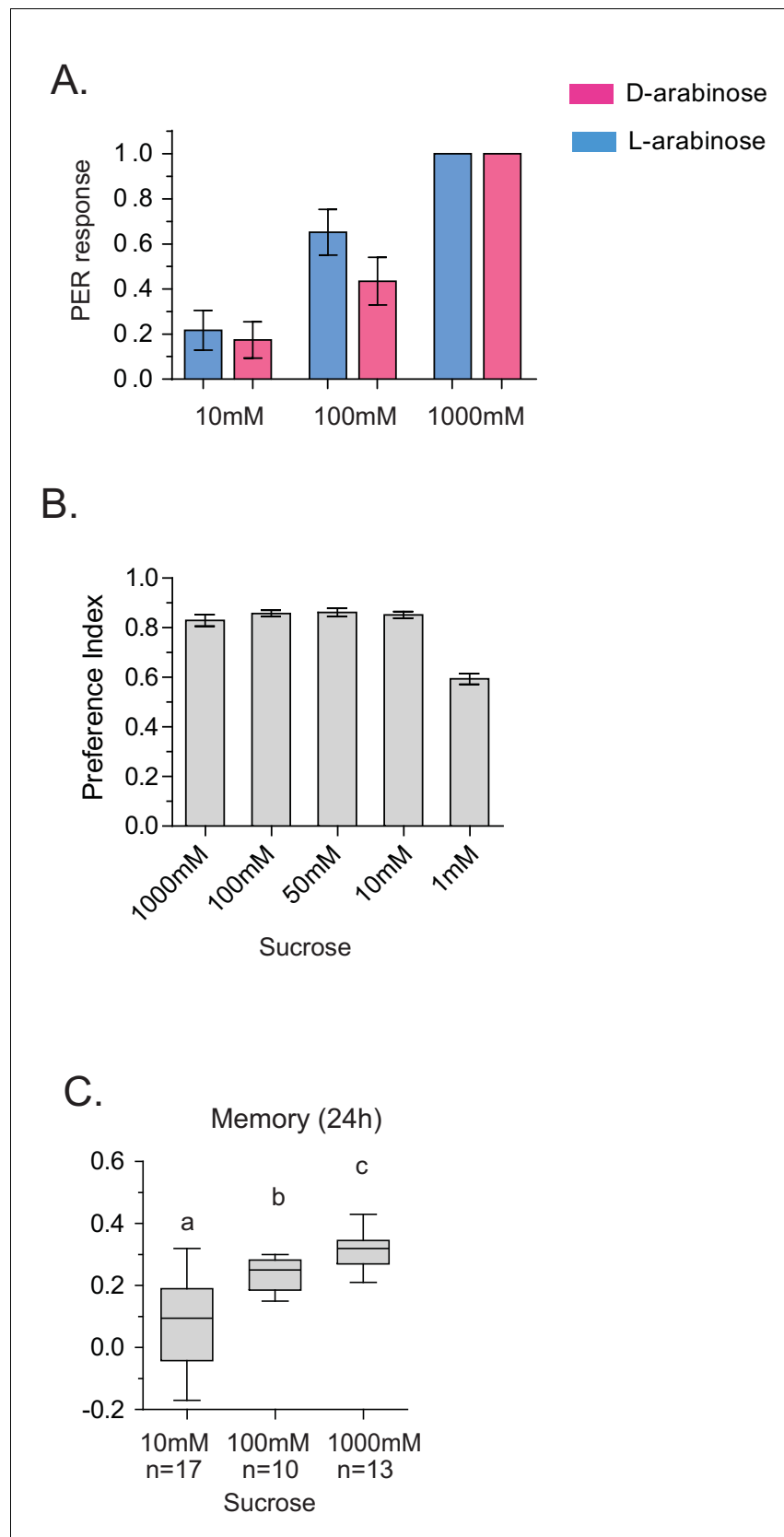


Figure 2—figure supplement 4. Detection and memory. (A) Proboscis extension reflex response to D- and L-arabinose. Flies were given a drop of water, then 10 mM D-ara, then 100 mM D-ara, then 1 M D-ara, water, Figure 2—figure supplement 4 continued on next page

Figure 2—figure supplement 4 continued

followed by 10 mM L-ara, 100 mM L-ara, and 1 M L-ara. Alternate flies were given D-ara or L-ara first. ($n = 26$ for each concentration). (B) Flies only begin to have trouble detecting sucrose in the two-choice test (paired with water) when sucrose is ≤ 1 mM. (C) Flies form better memories with increasing concentrations of sucrose. 10 mM sucrose (the maximum possible contamination given L-arabinose purity of $\geq 99\%$) does not produce robust long-term memory. For multiple samples, one-way ANOVA with Tukey's multiple comparisons test was performed, and significant differences ($p < 0.05$) are denoted by different letters. Results with error bars are means \pm s.e.m. ns, not significant. * ≤ 0.01 , ** ≤ 0.001 and *** ≤ 0.0001 .

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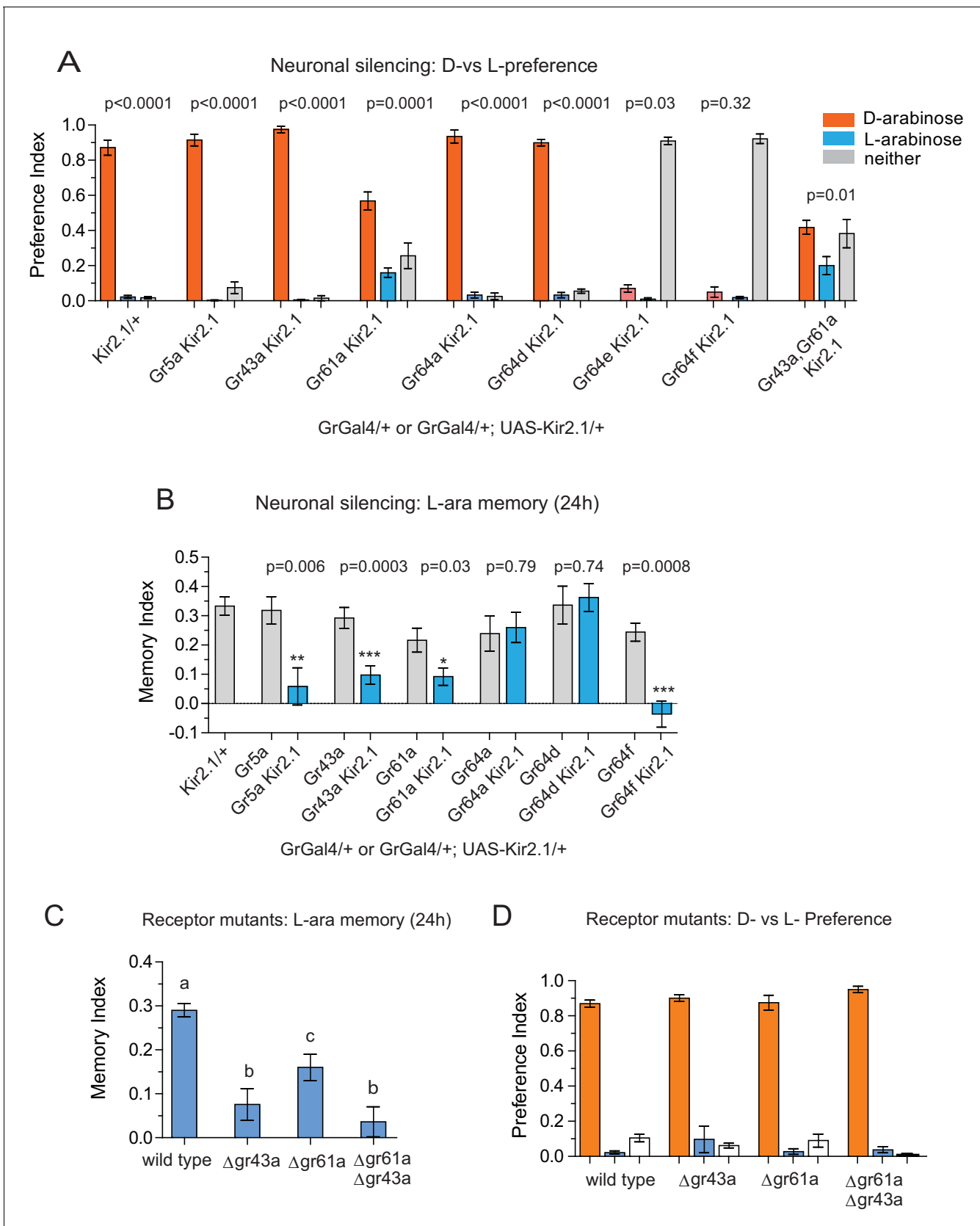


Figure 3. Gr43a- and Gr61a-expressing neurons are involved in D vs L preference and in L-arabinose memory. (A) Silencing of Gr61a-expressing neurons with Kir2.1 impaired D > L discrimination and preference; silencing Gr64e- and Gr64f-expressing neurons nearly eliminated detection of both Figure 3 continued on next page

Figure 3 continued

sugars. (B) Silencing Gr43a- and Gr61a-expressing neurons impaired L-arabinose memory. Gr64aGAL4 and Gr64dGAL4, whose expression is restricted to LSO and VCSC neurons did not impair L-arabinose memory. Silencing Gr64f- and Gr5a-neurons reduce L-arabinose memory, but they also impair L-arabinose detection. (see **Figure 3—figure supplement 1**). (C) Gr43a and Gr61a receptors are important for L-arabinose memory. (D) No single receptor mutant impaired D > L preference. For multiple samples, one-way ANOVA with Tukey's multiple comparisons test was performed, and significant differences ($p < 0.05$) are denoted by different letters. Results with error bars are means \pm s.e.m. ns, not significant. * ≤ 0.01 , ** ≤ 0.001 and *** ≤ 0.0001 .

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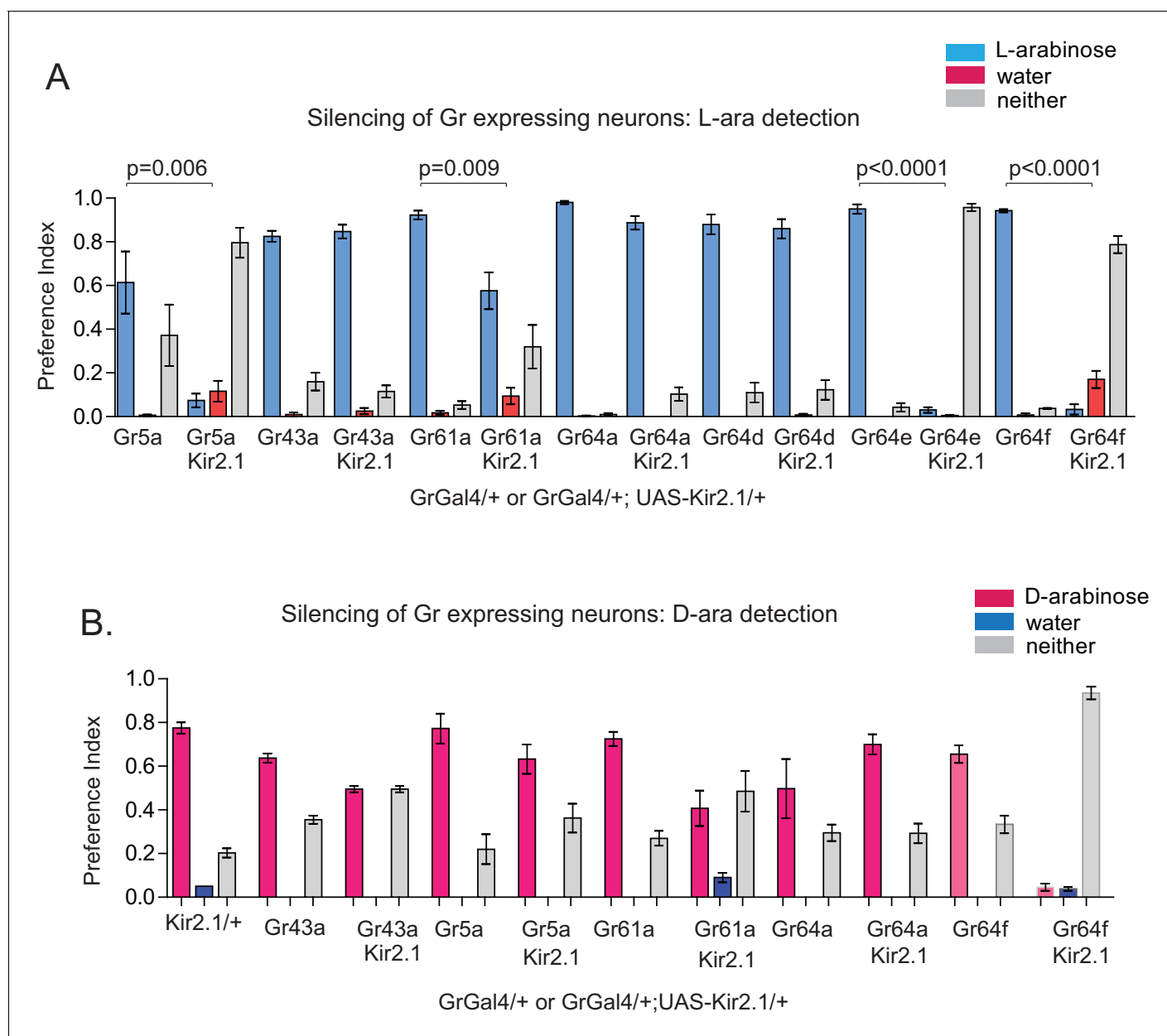


Figure 3—figure supplement 1. Gr expressing neurons involved in D- and L-arabinose detection. (A) Fifty male flies per trial were given water alone for 24–36 hr, then put on a microtiter plate checkered with food-dye-labeled water and 1 M L-arabinose. After 5 min, flies were removed and the color visible in the abdomen was scored. $n = 4$ for all silencing and mutant two-choice experiments, 50 flies per n . Silencing Gr5a-, Gr64e-, and Gr64f-expressing neurons nearly eliminated L-arabinose detection; silencing Gr43a^{GAL4} neurons had no detectable effect on L-arabinose detection. (B) Silencing Gr64f-expressing neurons virtually eliminates D-arabinose detection; silencing of Gr61a- and Gr43a-expressing neurons reduces but does not eliminate D-arabinose detection compared to the corresponding GAL4/+ control.

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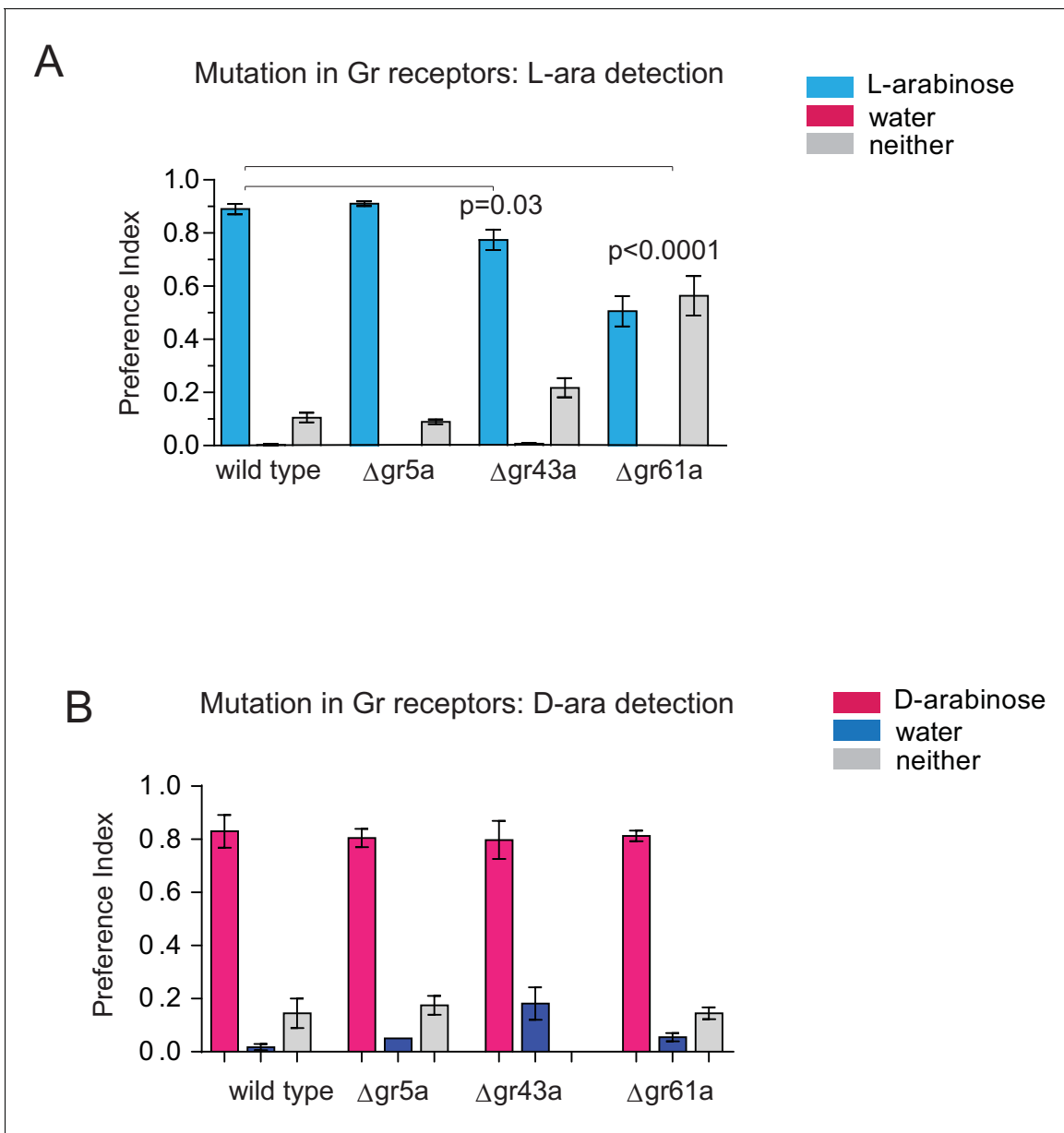


Figure 3—figure supplement 2. L- and D-arabinose detection do not rely on any single receptor. (A) Gr43a mutants have a small L-arabinose detection deficit while Gr61a mutants have a moderate detection deficit. (B) No single receptor removal impairs D-arabinose detection. Results with error bars are means \pm s.e.m.

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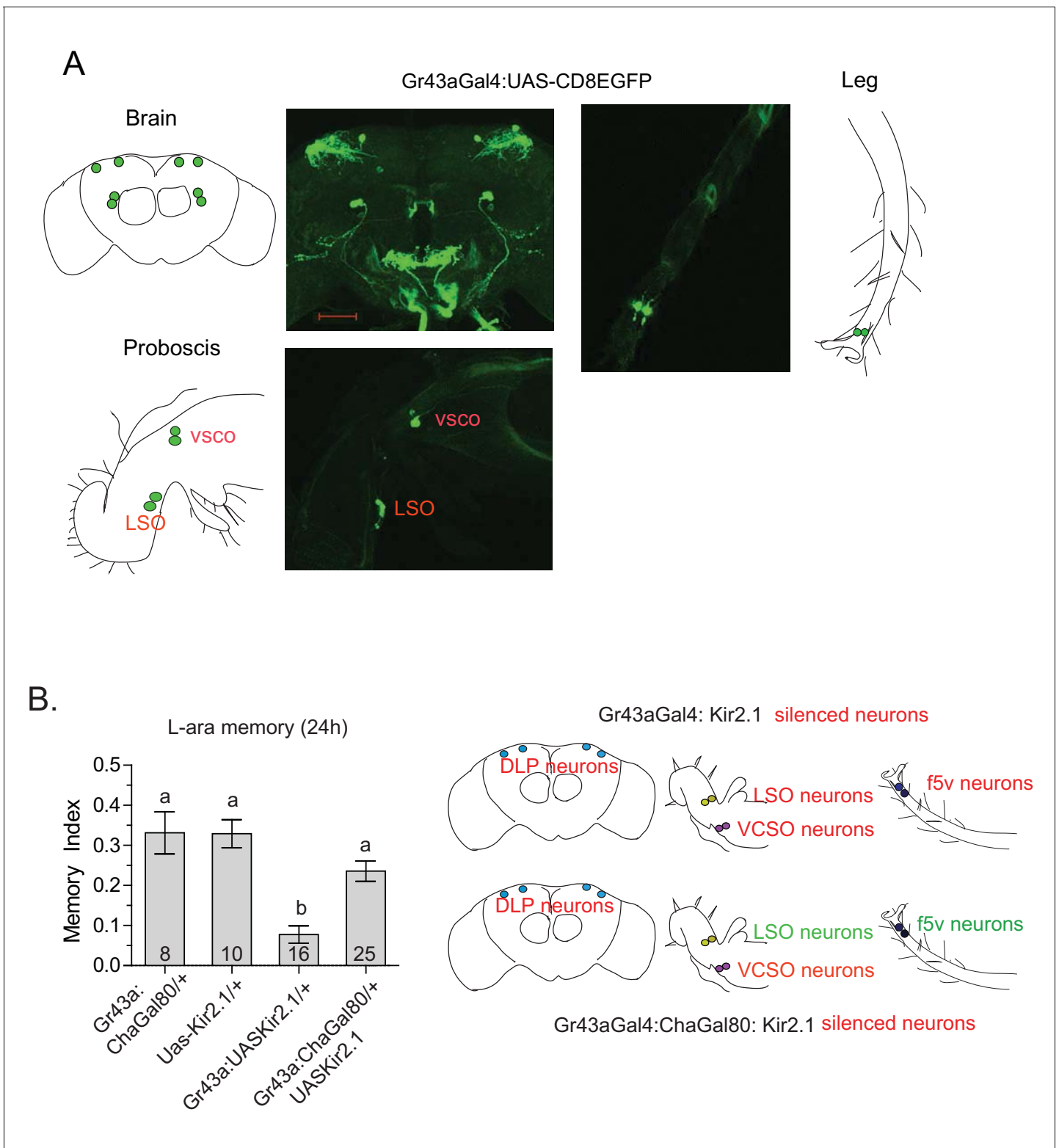


Figure 4. Tarsal Gr43a neurons are critical for L-arabinose memory. (A) Gr43a^{GAL4} neurons are observed in the dorsolateral protocerebrum, central brain, proboscis, leg, and gut (not shown). (B) Silencing only the dorsal protocerebral (DLP) and VCSO neurons does not impair L-arabinose memory. Left panel: memory score in various genetic backgrounds. Right panel, top: in Gr43a^{GAL4}/+; Kir2.1/+ flies, all indicated neurons are silenced (including pre-ventricular neurons, not pictured). Bottom: in Gr43a^{GAL4}:ChaGal80/+; UAS-Kir2.1/+ flies, only the neurons indicated in red type are silenced. For Figure 4 continued on next page

Figure 4 continued

multiple samples, one-way ANOVA with Tukey's multiple comparisons test was performed, and significant differences ($p < 0.05$) are denoted by different letters. Results with error bars are means \pm s.e.m. ns, not significant. * ≤ 0.01 , ** ≤ 0.001 and *** ≤ 0.0001 .

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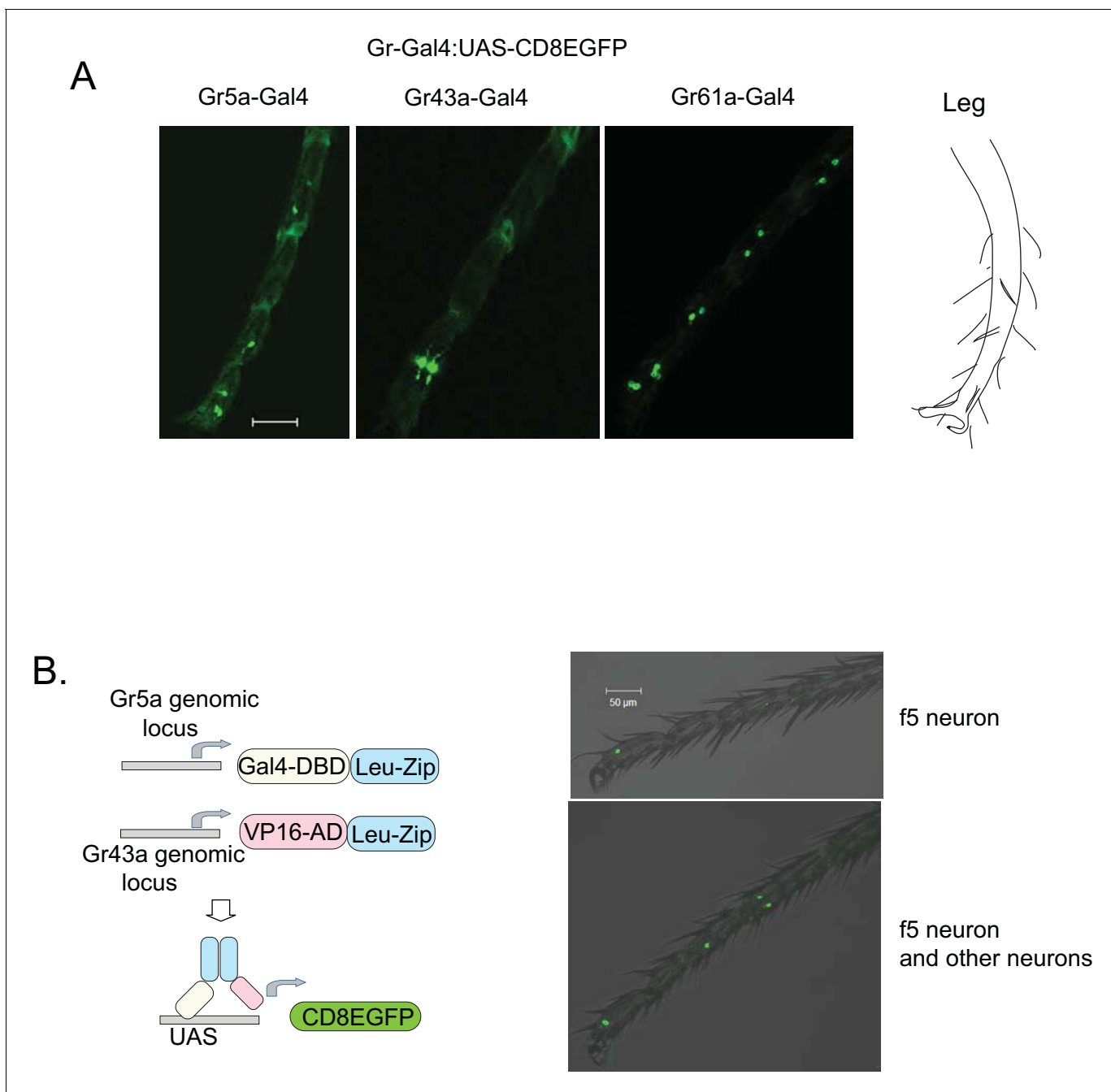


Figure 4—figure supplement 1. Expression patterns of Gr-GAL4s. (A) Distal tarsi expression patterns of Gr5aGAL4 (3 neurons), Gr43a^{GAL4} (2 neurons), and Gr61aGAL4 (6 neurons). Scale bar 50 μ m. (B) Gr5a-Gr43a splitGal4 labeling (Pfeiffer et al., 2010). GAL4 DNA-binding domain (DBD) and VP16 transcription activation domain (AD) fused to leucine zipper dimerization domains (Leu-Zip) were inserted into the Gr5a (DBD) and Gr43a (AD) genomic locus using CRISPR-Cas9. The neurons coexpressing both receptors drive mCD8eGFP from the UAS promoter. f5 neurons in the distal tarsi are marked by this technique. In some animals, an f5 neuron is the only neuron marked (top panel). However, in other animals, additional neurons were also marked in the leg (bottom panel) and proboscis. Scale bar 50 μ m.

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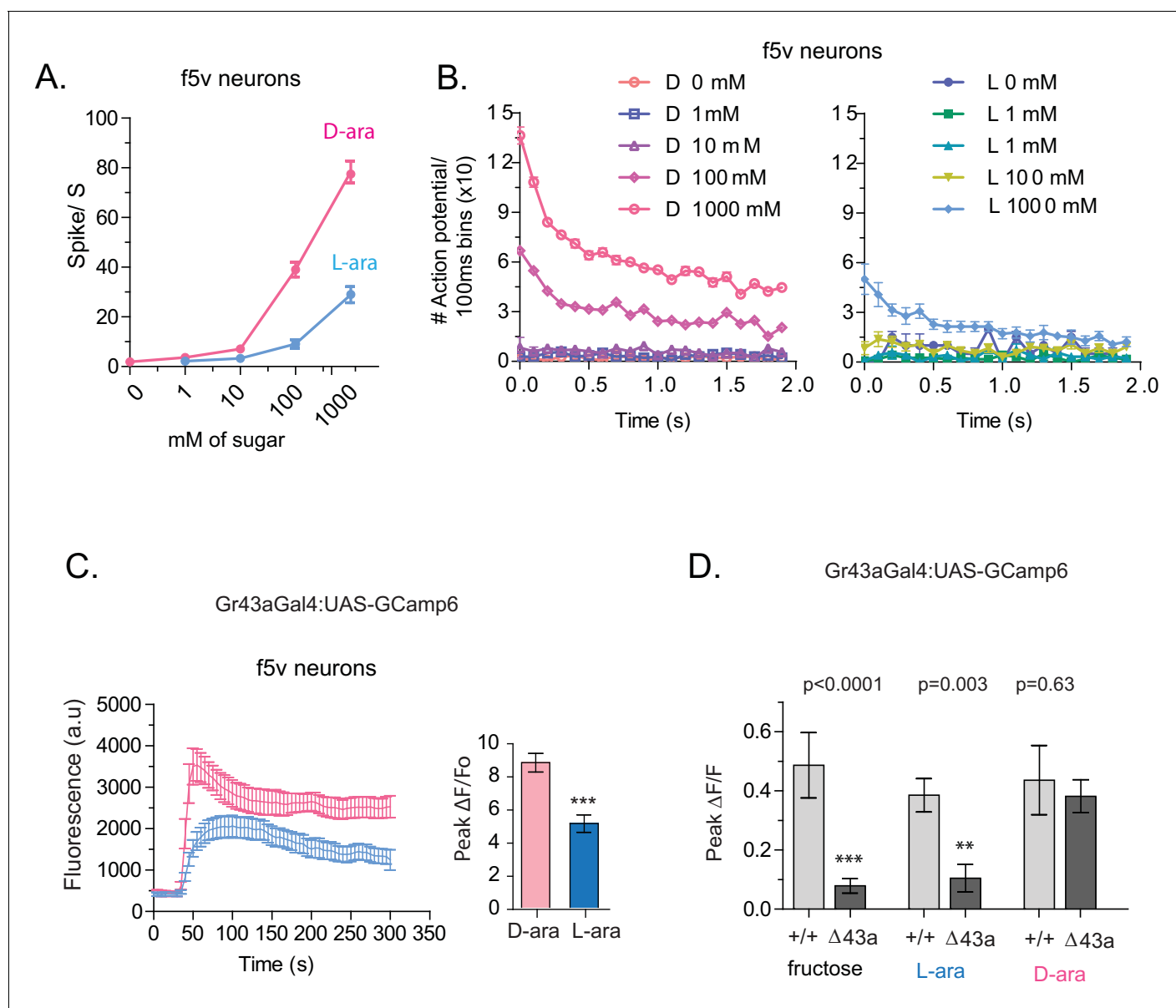


Figure 5. Tarsal Gr43a neurons respond differentially to D- and L-arabinose. (A) Spikes per second of recorded f5V tarsal neuron in response to D- or L-arabinose at various concentrations. (B) Spikes per second binned by 100 ms over the first two seconds of response. (C) The evoked-calcium activity of Gr43a^{GAL4} neurons in the distal tarsi. (D) Removal of Gr43a receptor impairs fructose and L-arabinose activation of Gr43a^{GAL4} neurons. Results with error bars are means \pm s.e.m. ns, not significant. * \leq 0.01, ** \leq 0.001 and *** \leq 0.0001.

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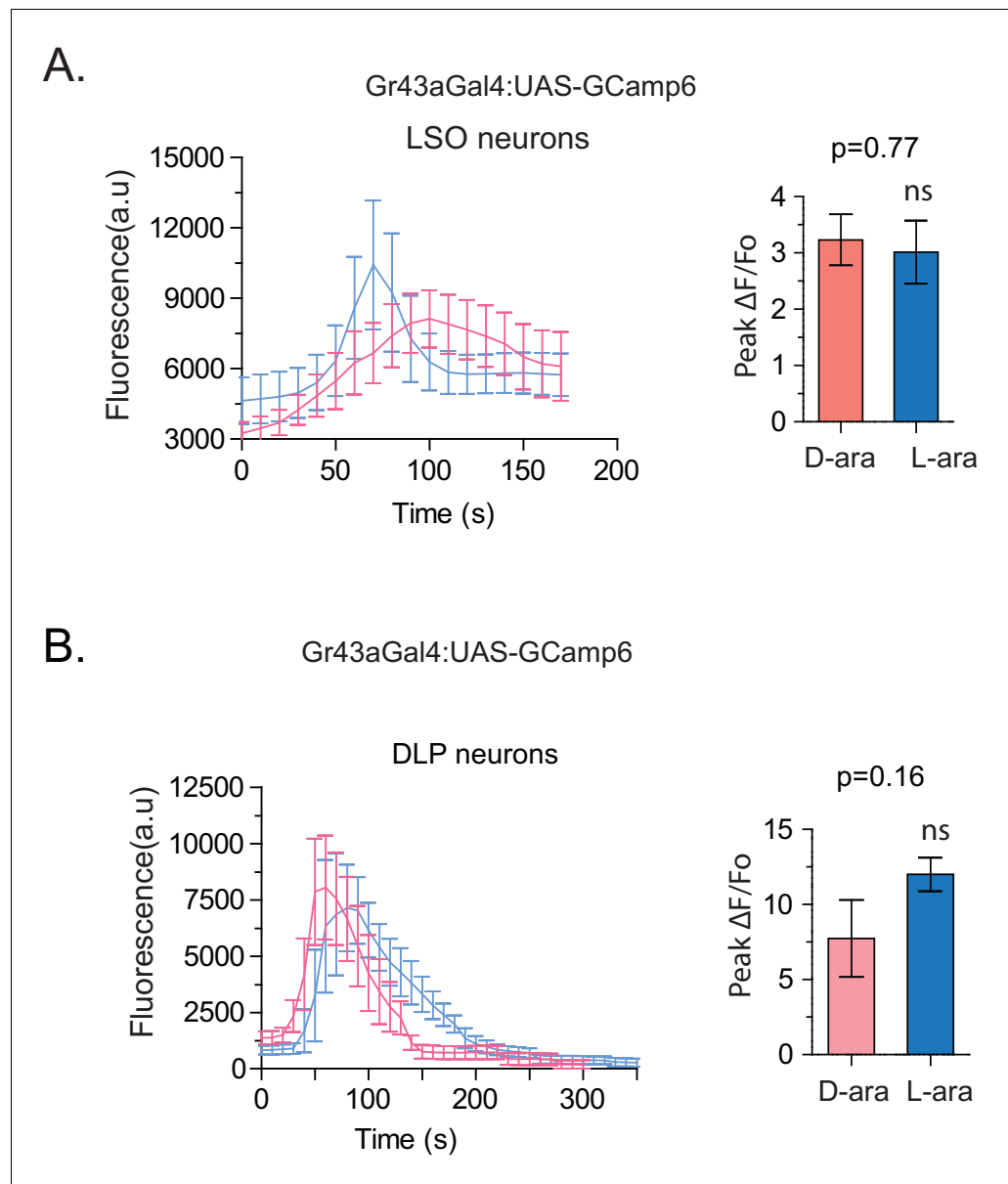


Figure 5—figure supplement 1. Evoked-calcium activity of Gr43a neurons in response to D- and L-arabinose. (A) Imaging of calcium responses of LSO neurons in proboscis. The left panel shows curve-aligned fluorescence to compare the shape of the responses, and the right panel plots peak magnitudes as $\Delta F/F_0$. (B) Imaging of calcium responses of dorsal protocerebral neurons. The left panel shows curve-aligned fluorescence to compare the shape of the responses, and the right panel plots peak magnitudes as $\Delta F/F_0$. Results with error bars are means \pm s.e.m. ns, not significant. * ≤ 0.01 , ** ≤ 0.001 and *** ≤ 0.0001 .

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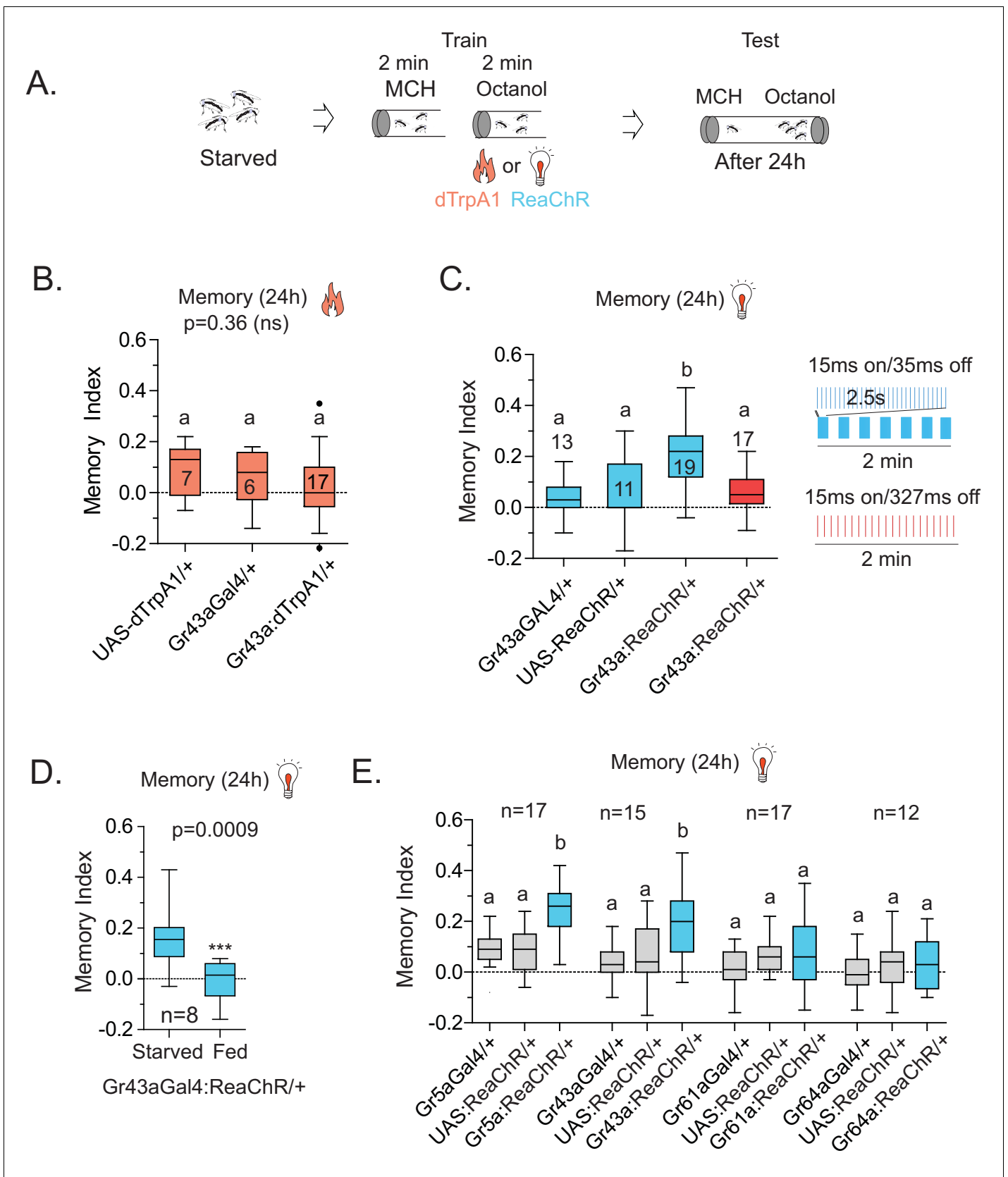


Figure 6. Activation of Gr43a neurons is sufficient to form rewarding associative memory. (A) Schematic of heat and light-activated associative olfactory training. (B) Activation of Gr43aGAL4 neurons by dTrpA1 (at 31 °C) does not induce long-term memory. (C) A 20 Hz, 15 ms pulse-width activation for 2.5 s, repeated every 20 s, induces long-term memory in flies expressing ReaChR in Gr43a^{GAL4} neurons; genetic controls do not show significant memory, and the same amount of light using the same pulse-width but distributed uniformly over the 2 min generates no memory (red). Schematics of light patterns are not to scale. (D) Optogenetic activation of Gr43a^{GAL4} neurons induces memory only in hungry flies, not in flies fed ad libitum. (E) Optogenetic activation of Gr43a- and Gr5a-expressing neurons leads to substantial 24 hr memory; activation of Gr61a- or Gr64a-expressing neurons does not. For multiple samples, one-way ANOVA with Tukey's multiple comparisons test was performed, and significant differences ($p < 0.05$) are denoted by different letters. Results with error bars are means \pm s.e.m. ns, not significant. * ≤ 0.01 , ** ≤ 0.001 and *** ≤ 0.0001 .

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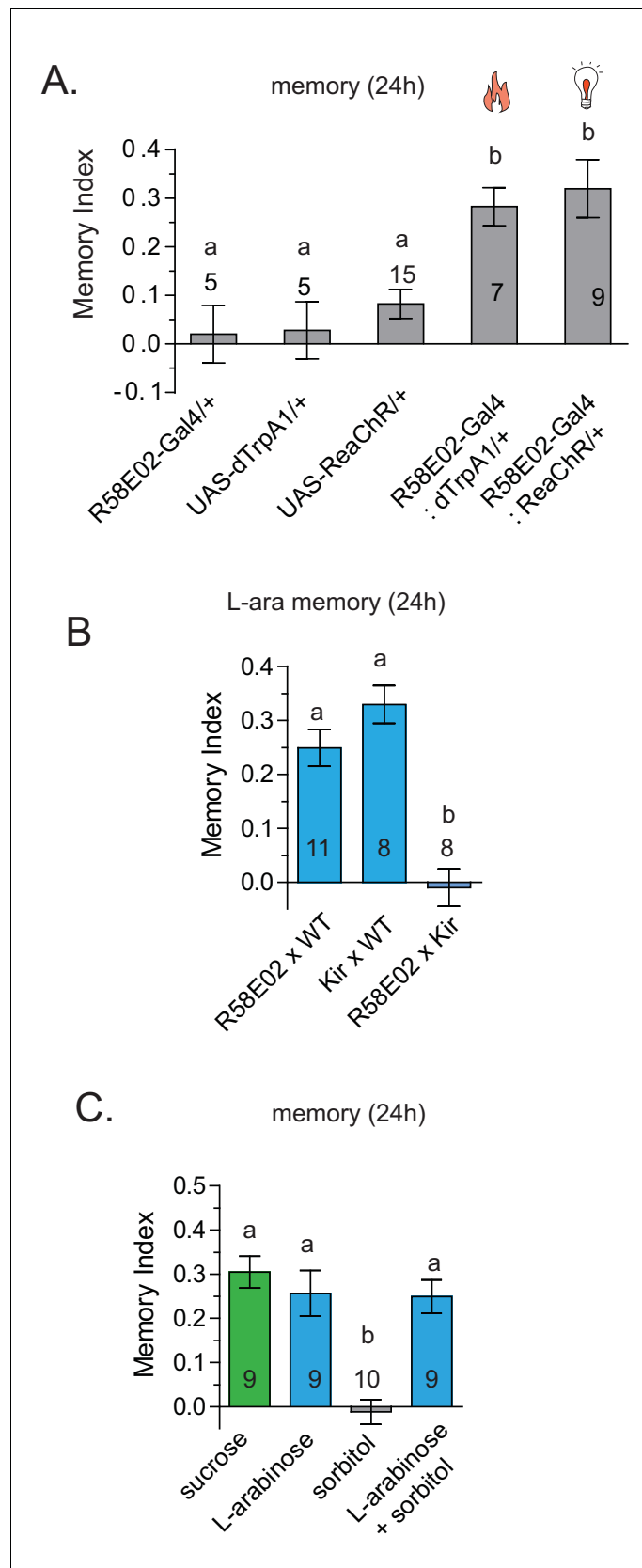


Figure 6—figure supplement 1. dTrpA1 activation of R58E02 neurons does not produce long-term memory. (A) Activation of R58E02GAL4 dopaminergic neurons, either with light (ReaChR; 1 Hz, 500 ms pulse-width for 20 s, Figure 6—figure supplement 1 continued on next page

Figure 6—figure supplement 1 continued

repeated three times over 2 min, or 10 Hz, 10 ms pulse-width, repeated continuously for 2 min; data combined) or with heat (dTrpA1; 31 °C for two minutes) is sufficient to generate robust 24 hr memory. **(B)** Silencing of R58E02GAL4 neurons eliminates 24 hr L-arabinose memory. **(C)** Supplementing L-arabinose with 1 M sorbitol does not increase L-arabinose memory. For multiple samples, one-way ANOVA with Tukey's multiple comparisons test was performed, and significant differences ($p < 0.05$) are denoted by different letters. Results with error bars are means \pm s.e.m. ns, not significant. * ≤ 0.01 , ** ≤ 0.001 and *** ≤ 0.0001 .

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G. Article 7 : *Drosophila* bitter taste(s)

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Abstract

Most animals possess taste receptors neurons detecting potentially noxious compounds. In humans, the ligands which activate these neurons define a sensory space called “bitter”. By extension, this term has been used in animals and insects to define molecules which induce aversive responses. In this review, based on our observations carried out in *Drosophila*, we examine how bitter compounds are detected and if bitter-sensitive neurons respond only to molecules bitter to humans. Like most animals, flies detect bitter chemicals through a specific population of taste neurons, distinct from those responding to sugars or to other modalities. Activating bitter-sensitive taste neurons induces aversive reactions and inhibits feeding. Bitter molecules also contribute to the suppression of sugar-neuron responses and can lead to a complete inhibition of the responses to sugar at the periphery. Since some bitter molecules activate bitter-sensitive neurons and some inhibit sugar detection, bitter molecules are represented by two sensory spaces which are only partially congruent. In addition to molecules which impact feeding, we recently discovered that the activation of bitter-sensitive neurons also induces grooming. Bitter-sensitive neurons of the wings and of the legs can sense chemicals from the gram negative bacteria, *Escherichia coli*, thus adding another biological function to these receptors. Bitter-sensitive neurons of the proboscis also respond to the inhibitory pheromone, 7-tricosene. Activating these neurons by bitter molecules in the context of sexual encounter inhibits courting and sexual reproduction, while activating these neurons with 7-tricosene in a feeding context will inhibit feeding. The picture that emerges from these observations is that the taste system is composed of detectors which monitor different “categories” of ligands, which facilitate or inhibit behaviors depending on the context (feeding, sexual reproduction, hygienic behavior), thus considerably extending the initial definition of “bitter” tasting.



Drosophila Bitter Taste(s)

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Most animals possess taste receptors neurons detecting potentially noxious compounds. In humans, the ligands which activate these neurons define a sensory space called “bitter”. By extension, this term has been used in animals and insects to define molecules which induce aversive responses. In this review, based on our observations carried out in *Drosophila*, we examine how bitter compounds are detected and if bitter-sensitive neurons respond only to molecules bitter to humans. Like most animals, flies detect bitter chemicals through a specific population of taste neurons, distinct from those responding to sugars or to other modalities. Activating bitter-sensitive taste neurons induces aversive reactions and inhibits feeding. Bitter molecules also contribute to the suppression of sugar-neuron responses and can lead to a complete inhibition of the responses to sugar at the periphery. Since some bitter molecules activate bitter-sensitive neurons and some inhibit sugar detection, bitter molecules are represented by two sensory spaces which are only partially congruent. In addition to molecules which impact feeding, we recently discovered that the activation of bitter-sensitive neurons also induces grooming. Bitter-sensitive neurons of the wings and of the legs can sense chemicals from the gram negative bacteria, *Escherichia coli*, thus adding another biological function to these receptors. Bitter-sensitive neurons of the proboscis also respond to the inhibitory pheromone, 7-tricosene. Activating these neurons by bitter molecules in the context of sexual encounter inhibits courting and sexual reproduction, while activating these neurons with 7-tricosene in a feeding context will inhibit feeding. The picture that emerges from these observations is that the taste system is composed of detectors which monitor different “categories” of ligands, which facilitate or inhibit behaviors depending on the context (feeding, sexual reproduction, hygienic behavior), thus considerably extending the initial definition of “bitter” tasting.

Keywords: taste, insects, aversive, pheromones, electrophysiology, behavior

INTRODUCTION

In humans, bitter taste is defined as a sensation associated with the perception of potentially toxic molecules such as alkaloids, which induce innate aversive reactions (Ventura and Worobey, 2013). Innate aversions can be subsequently reversed, and bitter tasting foods can even become appealing for example when post-ingestive effects are positive either physiologically or socially (Calabrese, 2008). Molecular studies support the view that bitter taste is mediated in vertebrates by specific receptor proteins Tas2Rs (Mueller et al., 2005; Meyerhof et al., 2011; Barretto et al., 2015), which are expressed within a specific population of taste sensory cells.

Activating these taste cells either by genuine ligands or through optogenetics, triggers aversive reactions (Chen et al., 2011). By extension, bitter sensation is inferred in other animals, even in insects, since the activation of specific taste cells triggers aversive reactions often associated with feeding and serves to protect individuals from accidental ingestion of noxious molecules.

Toxic molecules are used in numerous species of all taxon including plants, animals, insects and microorganisms as a defense against their predators (Berenbaum, 1995; Skelhorn and Rowe, 2009). Such molecules encompass a bewildering array of chemical structures (Lunceford and Kubanek, 2015). Many of them are toxic to the consumer, and a number of them are deterrent or repellent (Kool, 2005). For consumers, it makes sense to be able to detect protected preys and to avoid feeding from sources contaminated with toxic or noxious molecules. Animals which exploit resources with low quantities of toxic molecules tend to lose their bitter receptors (Li and Zhang, 2014) as in whales (Feng et al., 2014) or vampire bats (Hong and Zhao, 2014). Specialist animals tend to have low numbers of bitter receptors while generalist animals tend to have more of them (McBride, 2007; McBride and Arguello, 2007). There are exceptions to this general hypothesis: for example, the silkworm *Bombyx mori* is an absolute specialist as it feeds and develops exclusively on leaves of the mulberry tree but its repertoire of taste receptors shows an expansion of bitter receptors (Wanner and Robertson, 2008). Inversely, the honeybee *Apis mellifera* which is a generalist, has a low number of gustatory receptors (Robertson and Wanner, 2006). These contradictions may resolve if one wants to consider not the chemistry of the molecules, but their biological role. For *B. mori*, it is possible that the expansion of gustatory receptors allow them to recognize secondary compounds associated with their specific host plant. For *A. mellifera*, it is possible that their food resource has a composition that limits the risks of being exposed to noxious molecules.

These observations suggest nevertheless that all organisms have evolved a taste modality that allows them to detect and to avoid molecules which represents a potential danger. This taste modality is defined both by an ensemble of taste receptor genes that define a “bitter” space, and by populations of receptor cells expressing members of this family of receptors. In this paper, we want to review recent evidence drawn mostly from our own experience in *Drosophila* that cells sensitive to bitter compounds react to classes of molecules important in different behavioral contexts, and stress that bitter molecules also have an impact on the detection of other molecules detected through other taste modalities.

CONTACT CHEMORECEPTION IN *DROSOPHILA* ADULTS

Taste detection in *Drosophila* adults involves external and internal contact chemoreceptive sensilla which are distributed all over the body, especially in the oral region (proboscis and hypo- and epipharyngeal organs of the anterior digestive tract), on the legs, and on the front margins of the wings

(Stocker, 1994; Shanbhag et al., 2001; Isono and Morita, 2010). Contact chemoreceptive sensilla have a pore at their tip, while olfactory sensilla have tiny pores all over the shaft (Altner and Prillinger, 1980; Stocker, 1994). Most of these taste sensilla house four gustatory neurons and a mechanosensitive neuron (Shanbhag et al., 2001). Some proboscis taste sensilla house only two taste neurons (Hiroi et al., 2004), while taste pegs which are located in rows between and on the lateral sides of the six pseudotracheal rows of the proboscis, house only one (Shanbhag et al., 2001). The cellular organization of these sensory units with bipolar sensory cells and three types of accessory cells, is very similar to that of olfactory sensilla found on the antenna and the maxillary palps. However, while olfactory receptors neurons converge into glomeruli in the antennal lobe, taste receptor neurons project into neuropiles associated with each body segment and appendage (de Bruyne and Warr, 2006; Kwon et al., 2014), thus combining a chemotopic and a somatotopic map (Wang et al., 2004), whereas in other insects, either a clear somatotopic map exists as in *Schistocerca gregaria* (Newland et al., 2000) and *Periplaneta americana* (Nishino et al., 2005), or not as in *Phormia regina* (Edgecomb and Murdock, 1992).

Since the initial discovery of a family of putative gustatory receptor proteins (Clyne et al., 1999), continuous progresses have been made in elucidating molecular elements which enable gustatory receptor neurons (GRNs) to detect external chemicals. In *Drosophila melanogaster*, this family includes 60 genes which encode for 68 receptor proteins (Clyne et al., 2000; Dunipace et al., 2001; Scott et al., 2001; Robertson et al., 2003). These receptors are expressed in GRNs but also in other tissues such as the digestive tract, reproductive organs and epidermal cells on the abdomen (Park and Kwon, 2011a,b), into the brain (*Gr43a* and *Gr64a*; Miyamoto et al., 2012; Miyamoto and Amrein, 2014; Fujii et al., 2015), into the antenna either as receptors to CO₂ into specific sensilla (*Gr21a* and *Gr63a*; Jones et al., 2007; Yao and Carlson, 2010) or into olfactory neurons (*Gr5a*, *Gr64b* and *Gr64f*; Fujii et al., 2015) or even into multidendritic epithelial cells on the abdomen (*Gr66a*; Dunipace et al., 2001; Shimono et al., 2009). While GRs are generally thought to be involved in the detection of chemicals, they have been also shown to be involved in the detection of temperature (Ni et al., 2013).

GRNs express also a number of other genes which directly affect their sensitivity and selectivity. First of all, membrane-bound ionotropic receptors have been shown to affect pheromone and salt detection (Benton et al., 2009; Zhang et al., 2013a; Koh et al., 2014; Stewart et al., 2015). Transient receptor channels like TRPA1 and *pain* are involved in the detection of aversive molecules (Al-Anzi et al., 2006; Kim et al., 2010; Kwon et al., 2010), and pickpocket channels modulate pheromone and salt detection (Liu et al., 2003, 2012; Lin et al., 2005; Cameron et al., 2010; Chen et al., 2010; Lu et al., 2012; Pikielny, 2012; Starostina et al., 2012; Thistle et al., 2012; Toda et al., 2012; Alves et al., 2014). Taste sensitivity and selectivity is also modulated by proteins found in the sensillum lymph around the neurons such as odorant binding proteins (Galindo and Smith, 2001; Shanbhag et al., 2001; Koganezawa and Shimada, 2002; Park et al., 2006; Jeong et al., 2013), chemosensory

proteins like CheB (Xu et al., 2002; Park et al., 2006; Ben-Shahar et al., 2007, 2010; Starostina et al., 2009) and various enzymes such as sugar-hydrolyzing proteins (Bhavsar et al., 1983).

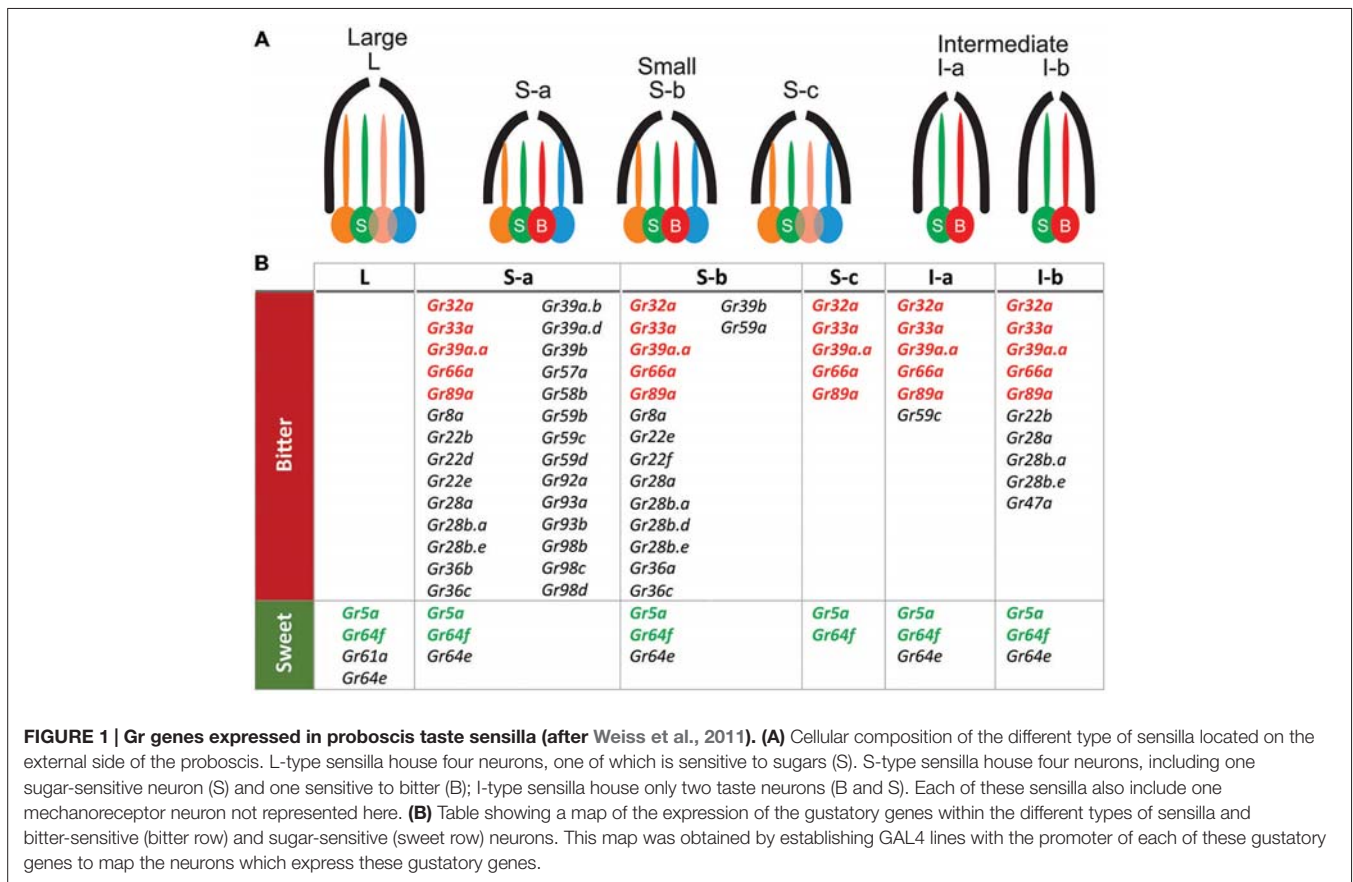
This impressive array of genes is by no means complete but the picture that emerges seems clearer when it comes to mapping their expression to specific populations of neurons. Earlier electrophysiological studies in *Drosophila* promoted the view that GRNs would fall in four functional categories, respectively sensitive to sugars, salt, bitter molecules and water (Fujishiro et al., 1984; Singh, 1997; Meunier et al., 2003). Many exceptions to this scheme were found in various insects, such as water-cells responding to sugars (Wieczorek and Köppl, 1978; Wieczorek, 1980), or salt cells responding to sugar or lactose (Schnuch and Hansen, 1990, 1992). The situation is even more confusing in phytophagous insects where establishing a terminology distinguishing prototypic cell types across species seems quite difficult (Chapman, 2003). This lead Bernays and Chapman (2001) to consider only two functional types of cells, called phago-stimulant and phago-deterrent.

In flies at least two groups of sensory cells can be distinguished on the basis of the receptors they express (Figure 1): sugar-sensitive cells which co-express several gustatory genes such as *Gr5a*, *Gr64a-f* and *Gr61a* (Dahanukar et al., 2001, 2007; Scott et al., 2001; Thorne et al., 2004; Jiao et al., 2007; Slone et al., 2007; Weiss et al., 2011; Fujii et al., 2015), and bitter-sensitive cells

which co-express several other gustatory genes such as *Gr66a*, *Gr33a* and *Gr93a* (Dunipace et al., 2001; Scott et al., 2001; Thorne et al., 2004; Moon et al., 2006; Weiss et al., 2011; Ling et al., 2014). Within these two categories, subtypes have been described both on the proboscis (Weiss et al., 2011) and on the legs (Ling et al., 2014), suggesting that flies may possess finer discrimination capabilities than currently thought (but see Masek and Scott, 2010).

It must be stressed that most of these observations rely upon the use of reporter genes using Gal4 or LexA enhancer trap systems (Brand and Perrimon, 1993; Lai and Lee, 2006; Miyazaki and Ito, 2010) as the level of expression of these genes is relatively low. This means that these data should be considered with caution. For example, the expression of *Gr64a* within sugar-sensitive GRNs has been recently challenged (Fujii et al., 2015) although previous studies had positively identified this gene as being expressed and involved in sugar perception in these GRNs (Dahanukar et al., 2007; Jiao et al., 2007, 2008). It is possible that these apparent discrepancies are not only due to limitations of the enhancer-trap approach, but also to differences of expression levels of these genes, depending on the genetic background or on the rearing conditions (Nishimura et al., 2012).

The current view is that several GR proteins are needed to make one functional receptor unit (Jiao et al., 2008; Lee et al., 2009, 2010). To be fully functional, a bitter receptor may need



the co-expression of *Gr32a*, *Gr33a*, *Gr66a* (Moon et al., 2009; Lee et al., 2010) as well as of *Gr89a* and *Gr39a* which may represent “core-bitter *Grs*” (Weiss et al., 2011). Besides these core receptors, additional receptors may have a more specific role in the detection of particular chemicals such as GR59c for berberine, lobeline and denatonium (Weiss et al., 2011) and GR47a for strychnine (Lee et al., 2015). Sugar receptors may have a different set of core receptors (Dahanukar et al., 2001, 2007; Chyb et al., 2003; Jiao et al., 2007; Slone et al., 2007; Wisotsky et al., 2011; Ling et al., 2014; Yavuz et al., 2014; Fujii et al., 2015). This might explain why expressing individual bitter GRs into sugar-sensitive GRNs (and reversely) has failed so far (Lee et al., 2009; Montell, 2009; Isono and Morita, 2010).

The distinction between sugar- and bitter-sensitive taste cells is maintained in the way these cells project into the brain, in two non-overlapping areas at least in the subesophageal ganglion (Wang et al., 2004; Marella et al., 2006; Miyazaki and Ito, 2010; Kwon et al., 2014; Harris et al., 2015). Activating one class of these receptors using ectopically expressed reporters triggers either appetitive or aversive behaviors (Wang et al., 2004; Marella et al., 2006; Hiroi et al., 2008; Harris et al., 2015).

The picture that emerges from these observations, however incomplete it might be, is that taste encoding in flies rests upon global categories or modalities such as appetitive or aversive (Thorne et al., 2004; Amrein and Thorne, 2005; Harris et al., 2015), in a way strikingly similar to what molecular studies have shown in vertebrates (Scott, 2005; Chandrashekar et al., 2006; Yarmolinsky et al., 2009; Chen et al., 2011; Liman et al., 2014; Barretto et al., 2015). The hypothesis that categories of receptors deal with different types of molecules inducing appetitive or aversive behaviors, does not match the view that emerged when recording from taste nerves in vertebrates, where no corresponding functional segregation could be made between fibers (Contreras and Lundy, 2000; Chen and Di Lorenzo, 2008; Frank et al., 2008). This latter encoding was called across fiber coding (Erickson, 2000, 2008a,b) as opposed to labeled lines coding. Actually, a similar inconsistency between peripheral recordings and the labeled line theory has been recently demonstrated in an insect, using multicellular recordings to monitor nerve activity and central responses in the subesophageal ganglion of taste sensilla from the proboscis of *Manduca sexta* adults (Reiter et al., 2015). These opposed views (labeled lines vs across-fiber encoding) are difficult to reconcile (Scott and Giza, 2000; Smith et al., 2000; de Brito Sanchez and Giurfa, 2011) as each theory is missing elements for a complete proof (Fox, 2008).

DIRECT DETECTION OF AVERSIVE MOLECULES

Specific Taste Cells are Activated by Bitter Molecules

Adult flies respond to a number of alkaloids and aversive molecules by reducing their feeding intake. This can be observed

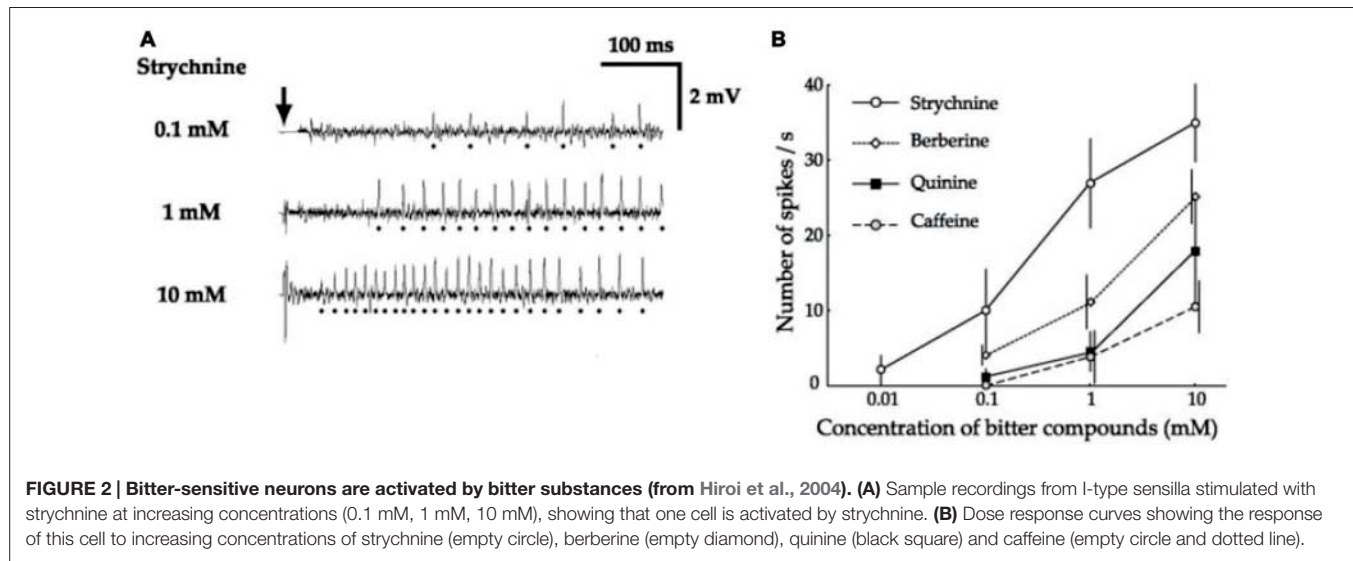
using a number of different behavioral tests: by monitoring the proportion of flies that have fed upon diets containing colored dyes (Tanimura et al., 1982; Meunier et al., 2003), by measuring the quantity of liquid ingested by flies (Ja et al., 2007; Sellier et al., 2011) or by monitoring the proboscis extension upon stimulation of the legs or proboscis (Meunier et al., 2003; Masek and Scott, 2010). For example, quinine which is bitter to humans and to many animals including insects, inhibits feeding in a dose-dependent way starting at 10^{-4} M when mixed with 35 mM fructose in agar (Meunier et al., 2003). Behavioral inhibition of the proboscis extension reflex occurs even when berberine (another alkaloid) is presented on one leg while the other leg is stimulated with sugar (Meunier et al., 2003).

Electrophysiological recordings indicated that this behavioral inhibition is correlated with the activation of specific cells, present in some sensilla of the legs (Meunier et al., 2003) and on the proboscis (Figure 2; Hiroi et al., 2004; Sellier, 2010; Sellier et al., 2011). Further observations coupled with selective expression of various reporter genes demonstrate that flies indeed have one class of cells responding to bitter compounds in a dose-dependent way. These cells co-express several gustatory receptors (up to 28; Weiss et al., 2011; Figure 1). These cells may also co-express receptors belonging to other classes, such as TRPA1 (Kim et al., 2010) or *painless* which confers them the capability to respond to aversive compounds such as wasabi (Al-Anzi et al., 2006), or even to respond to noxious temperature (Ni et al., 2013).

This population of cells which all express *Gr66a* on the proboscis, can be activated artificially, by expressing receptors responding to new stimuli such as capsaicin using the human vanilloid receptor VR1 (Marella et al., 2006), to light using the channel rhodopsin CHR2 (Zhang et al., 2007; Honda et al., 2014; French et al., 2015), or even to an odor, butyl acetate, using an olfactory receptor *Or22a* and *Orco* (Hiroi et al., 2008). These observations support the view that taste cells expressing gustatory receptors such as *Gr66a*, *Gr32a* and *Gr33a* detect a variety of bitter stimuli (Marella et al., 2006; Harris et al., 2015) and induce aversive behavioral responses such as feeding inhibition.

Bitter-Sensitive Taste Cells are Activated by Sex-Aversive Molecules

While contact chemoreceptors located all over the body are generally considered to function as detectors of sugars, bitter compounds, water and even salt, the detection of sexual pheromones is thought to be orchestrated by a group of specialized contact chemoreceptive sensilla. The distribution of these specialized sensilla is sexually dimorphic, whereby males have more taste sensilla on their legs (Nayak and Singh, 1983). During courtship, males go into several consecutive phases, one of which involves tapping on the abdomen of the females with their front legs (Spieth, 1974; Greenspan and Ferveur, 2000; Yamamoto and Koganezawa, 2013). Cobalt stainings showed that neurons from leg taste sensilla project differently



in males than in females (Possidente and Murphey, 1989). This situation is confirmed by the fact that pheromone detection by contact involves numerous molecular elements apparently not related to bitter-tasting such as CheB proteins (Xu et al., 2002; Park et al., 2006), *ppk23*, *ppk25* and *ppk29* DEG/Na channels (Lu et al., 2012; Pikielny, 2012; Thistle et al., 2012; Toda et al., 2012; Vijayan et al., 2014), gustatory receptors like *Gr39a*, *Gr32a* and *Gr68a* (Miyamoto and Amrein, 2008; Moon et al., 2009; Koganezawa et al., 2010; Wang et al., 2011; Watanabe et al., 2011), and ionotropic receptors (Koh et al., 2014).

However, very few studies have considered the wiring of these pheromone-sensitive cells, even though male-to-male detection is affected when “bitter” gustatory receptors such as *Gr32a* and *Gr38a* are inactivated (Miyamoto and Amrein, 2008; Moon et al., 2009). The involvement of *Gr32a* and *Gr38a* in pheromone detection is thought to be an indication that these *Grs* are obligatory co-receptors (Miyamoto and Amrein, 2008; Moon et al., 2009), in the same way as *Orco* (formerly known as *Or83b*) is an obligatory co-receptor in olfaction (Larsson et al., 2004). However, there is an even simpler explanation of the mixed roles of these *Grs* in the detection of pheromones and of bitter compounds, which is that aversive pheromones and bitter compounds may activate the same cells. We demonstrated on taste sensilla of the proboscis, that the same neuron responds both to caffeine and to 7-tricosene (7-T), which is a male inhibitory sexual pheromone (Figure 3). We further demonstrated that 7-T inhibits feeding while caffeine, berberine or quinine inhibit courtship (Lacaille et al., 2007). The simplest explanation of these observations is that the same neurons are used to detect different classes of signal, and that the central nervous system has limited capabilities to discriminate them. In other words, inhibitory pheromones taste “bitter” to flies.

Given the number of receptors expressed in this class of gustatory cells, *i.e.*, up to 28 *Grs*, TRP channels and IRs, it

is likely that we have not yet found all the ligands to which bitter-sensitive cells respond. While most substances tested so far belong either to chemicals which are bitter to humans such as plant-derived compounds and artificial molecules like denatonium, or which play a role in intraspecific communication such as 7-T, it is tempting to speculate that bitter-sensitive taste neurons of flies also detect chemicals from their enemies, (predators, parasitoid insects or entomopathogens), or from their competitors such as bacteria or fungi. For example, grooming reactions can be induced in flies both by quinine and by extracts from the gram negative bacteria, *Escherichia coli* (Yanagawa et al., 2014), that belong to an entirely different category of chemicals than alkaloids and bitter molecules.

INDIRECT DETECTION

While “bitter” molecules are detected by a specific class of gustatory cells, they might also interfere with the detection of molecules belonging to other modalities. Together with the activation of bitter-sensitive cells, sugar-sensing inhibition is considered as one of the major mechanisms by which plant secondary compounds exert antifeedant actions upon herbivores (Schoonhoven, 1982; Mitchell and Sutcliffe, 1984; Schoonhoven et al., 1992; Chapman, 2003). These inhibitions represent a “latent spectrum” as coined by Schoonhoven et al. (1992). Rather than being a curiosity or some kind of chemical artefact, we believe this mechanism represents an integral part of gustatory coding of bitter molecules in insects. Sugar-sensing inhibition by quinine for example has been observed very early in insects (Morita and Yamashita, 1959). In *Drosophila*, sugar-sensing inhibition (Siddiqi and Rodrigues, 1980), was described before bitter-sensitive cells were identified (Meunier et al., 2003).

Peripheral sugar-sensing inhibition seems a general phenomenon, as it occurs also in vertebrates

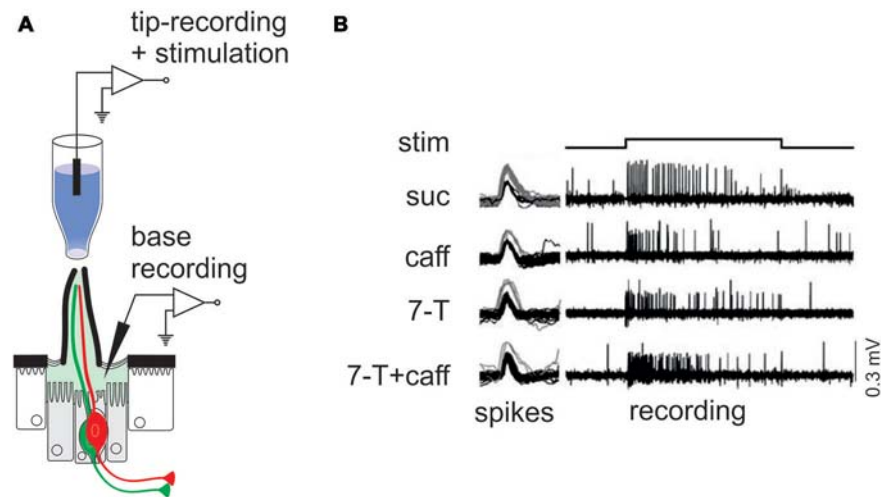


FIGURE 3 | Bitter-sensitive cells respond also to inhibitory sexual pheromones. (A) Diagram showing the two electrodes configuration used to record extracellular activities from taste sensilla of *Drosophila*. In all cases, a glass capillary containing the stimulus is used to cap the tip of a gustatory sensillum. If the stimulus is water-soluble, the stimulus electrode can contain an electrolyte and can be used to record electrical signals from the neurons within the sensilla. If the stimulus is lipophilic, the stimulus electrode which contains paraffin oil with the ligand, is no longer conductive and we use another electrode, for example a fine tapered tungsten rod, inserted at the base of a sensillum. **(B)** Sample recordings obtained from an I-type sensillum on the proboscis of *Drosophila* using a tungsten recording electrode, and stimulating either with sucrose (suc), caffeine (caff), 7-tricosene (7-T) or a mixture of 7-tricosene and caffeine (reproduced from Lacaille et al., 2007).

(Akaike and Sato, 1976; Ogawa et al., 1997; Frank et al., 2005) and in other organisms such as leeches (Li et al., 2001). In vertebrates, sugar-sensing inhibition by quinine has been attributed to the direct inhibition of TRPM5 (Talavera et al., 2008), but also to interactions with G proteins (Naim et al., 1994), to K^+ channels inhibition (Burgess et al., 1981) or even to the rapid entry into the cells inducing non-specific inhibition in taste cells (Peri et al., 2000). Thus far, no unitary mechanism explaining sugar-sensing inhibition by molecules such as quinine has been found. Bitter molecules may be detected either directly through a sensory receptor (not yet found), by interfering with the detection of sugar molecules via interaction with sugar receptors, or indirectly by interfering with or blocking various transduction elements.

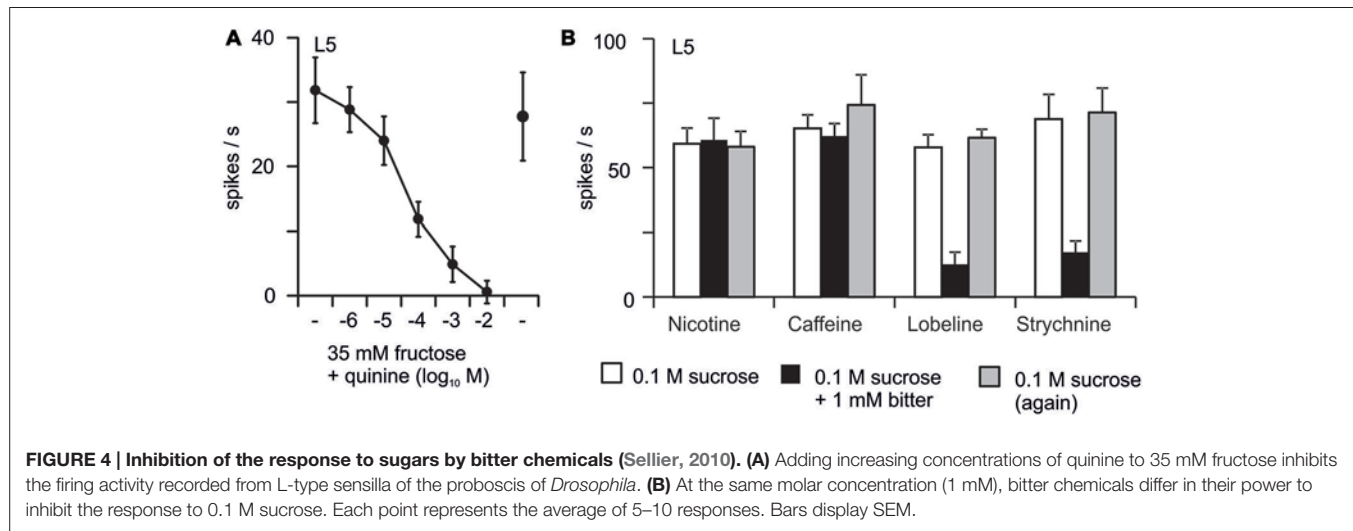
In *Drosophila*, sugar-sensing inhibition by bitter molecules can be demonstrated under at least two experimental situations. First, exposure to bitter chemicals may alter the detection of other tastants. For example, pre-exposing leg taste sensilla to 5 mM quinine during 10 s completely shuts down the response to sugar, and it takes 40 min to get a full recovery (Meunier et al., 2003). This inhibition might be due to a direct toxicity exerted upon nerve cells such as with vinblastine, colchicine (Matsumoto and Farley, 1978) or papain (Tanimura and Shimada, 1981), or it might be due to quinine molecules lingering in the sensillum lymph. Actually, as quinine is not prevalent in the environment of flies, they might miss proper degradation enzymes to clear the sensillum lymph. Secondly, bitter molecules may directly interfere with sugar detection (Sellier et al., 2011; French et al., 2015), either directly or indirectly, via an OBP (Jeong et al., 2013). Sugar-sensing inhibition differs between bitter chemicals (Figure 4; French et al., 2015), and between sugars

(Schoonhoven, 1982; Schoonhoven and Liner, 1994; Martin and Shields, 2012). Given the enormous range in the chemical structures of “bitter” chemicals, it is likely that a variety of modes of action will be found.

In addition to peripheral sensory inhibition involving a direct interaction of bitter molecules with sugar sensitive cells, bitter chemicals may interfere with gustatory perception through other pathways. One mechanism could be through lateral interactions between sensory cells, for example through ephaptic inhibition as demonstrated for olfactory cells (Su et al., 2012). Such mechanism was not found in the taste sensilla tested so far (French et al., 2015), but non-synaptic interactions are definitely relevant for gustation. Another mechanism involves higher-order circuits, such as presynaptic inhibition of sugar sensing neurons by bitter-sensitive neurons through GABA receptors (Chu et al., 2014). Given the importance of the gustatory system in triggering or preventing feeding, we certainly expect modulations to occur at the level of the sensory neurons as well as in the central circuitry decoding this information. Recent observations made it clear that satiety has a strong effect on how odors are decoded (Ko et al., 2015), and how appetitive or bitter tastants trigger feeding reactions (Inagaki et al., 2014). Likewise, mating alters strongly female food preferences to proteins (Ribeiro and Dickson, 2010) and possibly to bitter chemicals as well.

FUTURE PROSPECTS

All the data reported so far are compatible with the idea that bitter taste represents a well-defined taste modality which is different from sweet taste, at least when it comes to feeding.

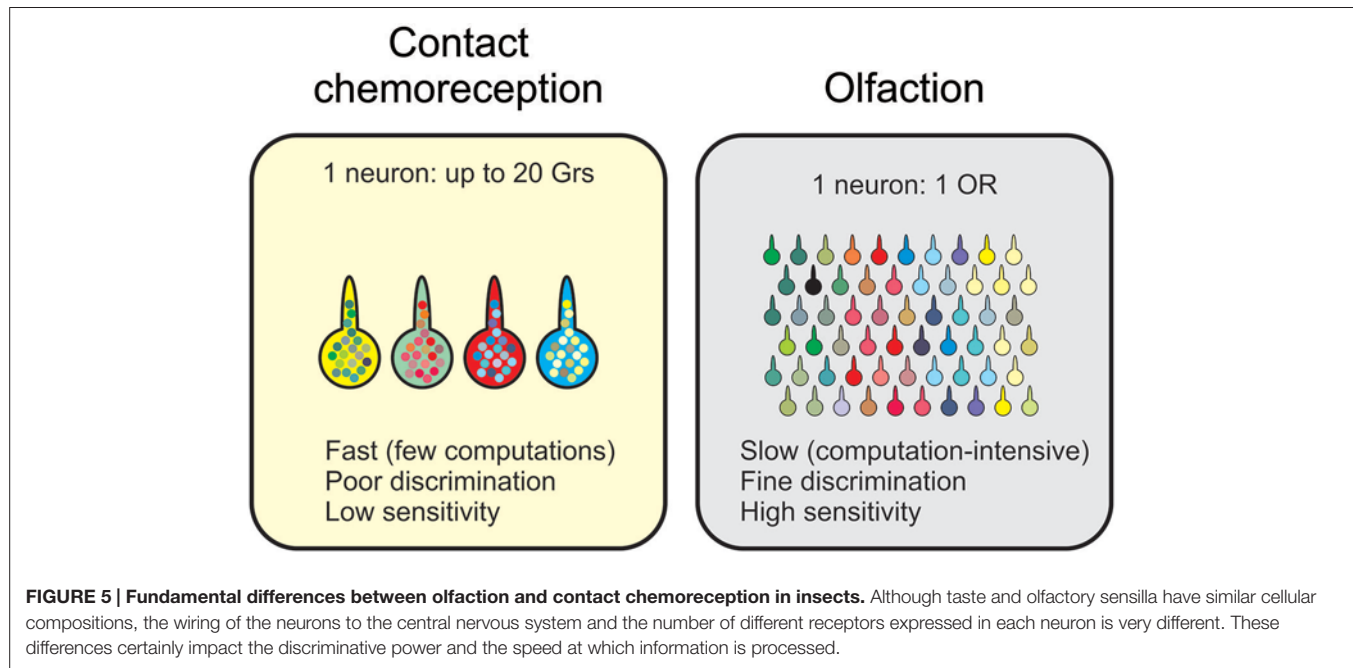


Bitter-sensitive cells are defined at a molecular level by the expression of a population of taste receptors, and activating these cells inhibits feeding. The behavioral inhibition is context-dependent, in that activating the same cells (on the proboscis) can either deter feeding or interfere with sex activities. This description is compatible with the view that insects may not be able to discriminate between different “bitter” molecules (Masek and Scott, 2010). Accordingly, the currently available data about how these neurons project in the central nervous system clearly indicate that bitter-sensitive neurons project to areas of the brain that are distinct from those where sugar-sensitive neurons project (Wang et al., 2004; Marella et al., 2006; Harris et al., 2015), maintaining the segregation observed at the periphery.

This might not be the last word of it, as sub-classes exist within the bitter modality (Weiss et al., 2011), and as taste neurons may encode bitter chemicals with different temporal codes (Glendinning et al., 2002, 2006) or even spatio-temporal codes (Reiter et al., 2015). However, even if one finds experimental evidence of rich encoding capabilities, so far, we are lacking clear behavioral evidences that flies can discriminate bitter molecules or bitter “categories”, independent of their concentration. Indications of such differences may come from looking more closely at different behaviors. For example, flies may prefer to lay eggs into food laced with bitter molecules (Yang et al., 2008; Schwartz et al., 2012; Dweck et al., 2013) instead of plain sugar (Yang et al., 2015), or into a medium rich in alcohol, especially if females were previously confronted with parasitoid wasps (Kacsoh et al., 2013, 2015). They might also change their natural preferences following larval exposure (Jaenike, 1982, 1983; Abed-Vieillard et al., 2014) or following the experience of others through social communication (Battesti et al., 2015). If not all “bitter” molecules are inducing aversive reactions in all behavioral contexts, this leaves open the possibility to test whether females can discriminate between different bitter molecules (but see Masek and Scott, 2010).

If the category “bitter” in flies regroup different shades or categories of bitterness, it seems to be pretty clear that the link between the noxiousness of molecules and their bitter taste is not a direct one. This lack of direct link has been clearly stated by Glendinning (1994, 2002, 2008), and has been experimentally tested in several phytophagous insects (Cottee et al., 1988; Usher et al., 1989; Bernays, 1990, 1991; Lee and Bernays, 1990; Bernays and Cornelius, 1992). This discrepancy between the intuitive role of bitterness to help avoiding intoxication and the lack of direct link between toxicity and bitterness should resolve if one considers aversive taste as a “correlation” established throughout evolution between a stimulus detected in the environment and a danger (or reduced fitness). One of the best examples for this comes from glucose-averse cockroaches (Silverman and Bieman, 1993) which avoid insecticide-treated diets, apparently through a mutation that allow resistant cockroaches to detect glucose (which is always associated with the insecticide) as a “bitter” molecule (Wada-Katsumata et al., 2013). Obviously, glucose is not toxic (Silverman, 1995; Silverman and Selbach, 1998), but it has become a signal for a toxic molecule in the environment.

Finally, it is striking to compare how information is analyzed in contact chemoreception and olfaction. Both systems are devoted to the detection of molecules in the external environment, using sensory receptors which are structured in a very similar way, with bipolar sensory cells enwrapped into accessory cells, sending dendrites into the sensillum lymph and their axon to the brain. However, the molecular logic and the wiring of the two systems are completely different. While the hedonic value of tastants seems to be determined already at the periphery with cells co-expressing a mosaic of receptors tuned to ligands pertaining to one or the other category, this distinction is less clear in olfaction (Knaden et al., 2012), as olfactory neurons express a very reduced set of receptors (Larsson et al., 2004; Goldman et al., 2005). This different structure probably imposes constraints on the functioning of the system, on its discriminative power, speed of decision and sensitivity threshold (Figure 5) as well as on its plasticity.



Olfaction applies a relatively fixed array of filters on the external world, and decoding this grid of filters is done through a network of interconnected neurons at the level of the antennal lobes and then in the lateral horn and the mushroom bodies. This arrangement leaves room for plasticity in how information is decoded, taking into account experience and both internal and external environmental conditions. The gustatory system on the other hand appears more rigid with a bitter and a sweet modality defined by groups of gustatory receptors expressed in different categories of cells. Such a system does not seem to leave much space to plasticity as regards the hedonic value of molecules, except by modulating their impact by amplifying or decreasing their detection at the level of the central nervous system where

a number of synaptic and neurohormonal regulations seem to occur, or directly at the level of the GRNs, which could modulate the level of expression of their different receptors (Zhang et al., 2013b).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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IX. Résumé & Abstract

Titre : Physiologie des récepteurs gustatifs chez la mouche de vinaigre (*Drosophila melanogaster*)

Mots clés : Drosophile, amer, sucre, L-canavanine, récepteurs gustatifs, comportement, électrophysiologie.

Résumé : Chez les animaux et en particulier les insectes, l'alimentation comprend une phase d'examen sensoriel qui précède l'ingestion, afin notamment d'éviter d'ingérer des substances toxiques. Cette détection fait intervenir des cellules spécialisées dans la détection de telles molécules, cellules qui sont généralement qualifiées de sensibles aux goûts « amers ». À l'aide d'observations électrophysiologiques et comportementales, nous avons abordé comment un insecte modèle, la drosophile, était capable de détecter des substances potentiellement toxiques mélangées à des sucres à l'aide de ses neurones gustatifs.

Dans une première partie, nous avons étudié la détection de la L-canavanine, qui est un acide aminé non protéique. Cette molécule est toxique pour l'homme comme pour les animaux car elle est confondue par le métabolisme avec un acide aminé, la L-arginine, et intégrée à sa place dans les protéines. En utilisant des constructions génétiques et en particulier le système UAS-Gal4, nous avons montré que la L-canavanine est détectée par des cellules gustatives qui expriment une protéine réceptrice GR66a, qui est impliquée dans la détection de nombreuses substances amères. Nous avons également montré que, contrairement à la caféine, la détection de L-canavanine nécessite des protéines Gα fonctionnelles.

Nous avons ensuite étudié les interactions sucré-amer. Dans un premier travail, nous avons montré que l'addition de L-canavanine une solution sucrée n'altérerait pas la détection des sucres, contrairement à la strychnine qui peut complètement supprimer la détection du sucre dans les cellules gustatives. Grâce à des ablations spécifiques des cellules détectant l'amer,

nous avons pu montrer que cette inhibition était une propriété intrinsèque des cellules sensibles aux sucres. Les cellules sensibles aux sucres auraient donc des sites récepteurs non identifiés, sensibles à certains ligands amers. Nous avons également abordé des interactions inverses, à savoir l'inhibition de la détection de substances amères par des sucres, en confrontant 4 substances amères (denatonium, berberine, caféine, umbellifère) à 12 sucres. Les observations que nous avons réalisées montrent que certains sucres exercent un effet inhibiteur sur la détection des molécules amères testées. En utilisant des outils génétiques permettant l'ablation des cellules sensibles aux sucres, nous avons montré que cette inhibition est une propriété intrinsèque des cellules sensibles à l'amer. Cependant, cet effet inhibiteur est loin d'être aussi efficace que l'inhibition des substances amères sur la détection des sucres.

Dans une dernière partie, nous avons évalué la modulation de la détection gustative à l'aide d'analogues d'une neuro-hormone, la leucokinine, connue pour ses effets sur la diurèse. Lorsqu'elle est mélangée à une solution sucrée, ces analogues inhibent la détection des sucres par les sensilles gustatives, à la fois chez le moustique *Aedes aegypti* et chez la drosophile.

La détection de substances « amères » par les cellules gustatives de drosophiles implique donc deux voies de codage : l'une, spécifique, concerne des cellules dédiées à la détection des substances amères ; l'autre, moins spécifique, affecte les cellules dédiées à la détection des sucres. De manière réciproque, ces cellules dédiées à la détection des molécules sont affectées par la présence de ligands sucrés. Le codage des informations gustatives à la périphérie est donc un phénomène plus complexe qui nécessite d'étudier plus précisément la détection de composés en mélanges.

Title : Physiology of gustatory receptor neurons in the fruit fly (*Drosophila melanogaster*)

Keywords : Drosophila, bitter, sweet, L-canavanine, taste receptors, electrophysiology, behavior.

Abstract : In most animals including insects, ingestion is preceded by a close examination of the food, for example in order to detect the presence of potentially noxious chemicals. This detection involves specialized gustatory cells, which are generally described as sensitive to "bitter" tastes. Using electrophysiology and behavioral observations, we studied how a model insect, *Drosophila melanogaster*, can detect potentially toxic substances (described here as "bitter") when mixed with sugar molecules, with their gustatory neurons. In a first part, we studied how L-canavanine is detected. L-canavanine is a pseudo amino acid, which is confounded with L-arginine by the metabolism. Proteins which include L-canavanine are non-functional and this compound is toxic for animals including insects. Using genetic constructions based on the UAS-Gal4 expression system, we showed that L-canavanine is detected by gustatory cells expressing a receptor protein, GR66a, which is specific to most cells capable of detecting bitter substances. We also showed that, contrary to caffeine, the detection of L-canavanine requires functional Gα_o proteins. Then, we studied some aspects of the detection of mixtures of sweet and bitter molecules. In a first approach, we contributed to establish that L-canavanine does not impact sugar detection, while other chemicals like strychnine completely inhibit sugar detection. By using the UAS-Gal4 system to ablate bitter-sensitive cells, we could demonstrate that such inhibition is a specific property of sugar-sensitive

cells. These cells should have thus receptors for bitter substances which have not been identified yet. We also examined the reverse interaction, which is a possible role of sweet molecules to inhibit the detection of bitter substances. We examined the detection of denatonium, berberine, caffeine and umbelliferone in the presence of 12 different sugars, using behavioral and electrophysiology observations. By using genetic construction to ablate sugar-sensitive cells, we found that the sugar inhibitory action is not due to the presence of sugar-sensitive cells. It should be noted, however that in our experimental conditions, this inhibitory action is less efficient than the inhibition of bitter upon sugar detection. In a last part, we examined the modulation of gustatory perception by analogs of leucokinine, which is a neuropeptide involved in the diuresis of insects. We show that these analogs, when mixed with sugars in solution, can inhibit sugar detection by gustatory sensilla, both in *Aedes aegypti* mosquitoes and in *Drosophila*.

The detection of bitter molecules by gustatory neurons in *Drosophila* thus involves two main coding channels: one is specific, and involves gustatory cells dedicated to the detection of bitter molecules; the second one, less specific, is affecting cells which are dedicated to the detection of sugar molecules. Gustatory coding is thus a more complex phenomenon than previously thought on the basis of examining responses to single molecules, thus urging to study the responses of gustatory receptors to more complex and natural mixtures.