Tyramine and its *Amtyr1* receptor modulate attention in honey bees (*Apis mellifera*)

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The authors wish to dedicate this work to the memory of Professor Hans-Joachim Pflüger, who was a wonderful friend and a great colleague. He made significant contributions to understanding the roles of tyramine and octopamine in insect behavior.

Abstract: Animals must learn to ignore stimuli that are irrelevant to survival and attend to ones that enhance survival. When a stimulus regularly fails to be associated with an important consequence, subsequent excitatory learning about that stimulus can be delayed, which is a form of non-associative conditioning called 'latent inhibition'. Honey bees show latent inhibition toward an odor they have experienced without association with food reinforcement. Moreover, individual honey bees from the same colony differ in the degree to which they show latent inhibition, and these individual differences have a genetic basis. To investigate the mechanisms that underly individual differences in latent inhibition, we selected two honey bee lines for high and low latent inhibition, respectively. We crossed those lines and mapped a Quantitative Trait Locus (QTL) for latent inhibition to a region of the genome that contains the tyramine receptor gene *Amtyr1*[1]. We then show that disruption of *Amtyr1* signaling either pharmacologically or through RNAi qualitatively changes the expression of latent inhibition but has little effect on appetitive conditioning, and these results suggest that AmTYR1 modulates inhibitory processing in the CNS. Electrophysiological recordings from the brain during pharmacological blockade are consistent with a model that AmTYR1 indirectly regulates Hebbian plasticity at inhibitory synapses in the CNS. Finally, sequencing *Amtyr1* and its up and downstream genomic region for high and low line workers suggested that individual differences might arise from variation in transcriptional control rather than structural changes in the coding region. Our results therefore identify a distinct modulatory pathway for this type of non-associative learning, and we propose a model for how it modulates Hebbian plasticity at defined synapses in the CNS. We have shown elsewhere how this modulation also underlies potentially adaptive intracolonial learning differences among individuals that benefit colony survival.

Introduction

The ability to learn predictive associations between stimuli and important events, such as food or threats, is ubiquitous among animals (*1*), and it may underlie more complex

[1] We use *Amtyr1* to denote the gene and AmTYR1 the receptor throughout the text.

cognitive capabilities (*1, 2*). This ability arises from various forms of associative and operant conditioning(*3*). However, the absence of reward also provides important information for learning about stimuli, because all animals must use this information to redirect a limited attention capacity to more important stimuli (*4*). One important mechanism for learning to ignore irrelevant stimuli is called latent inhibition (*5*). After an animal is presented with a stimulus several times without reinforcement, learning is delayed or slower when that same stimulus is reinforced in a way that would normally produce robust excitatory conditioning. For example, when honey bees are repeatedly exposed to a floral odor without association to food rewards, their ability to subsequently learn an excitatory association of this odor with a reward is delayed or reduced (*6*). While many studies in the honey bee have focused around how the presence of reward shapes learning and memory (*7*), evaluating this important form of nonassociative learning has not received as much attention (*6, 8*). Yet, like in all animals, it plays an important ecological role in the learning repertoire of honey bees. The presence of unrewarding flowers in an otherwise productive patch of flowers (*9*), or the unreinforced presence of an odor in the colony (*10*), can influence foragers' choices of flowers during foraging trips.

Moreover, individual honey bees from the same colony differ in the degree to which they exhibit several learning traits (*11-15*), including latent inhibition(*12*). Several studies of different forms of learning have demonstrated that individual differences are heritable (*12*). Individuals showing different learning phenotypes occur within the same colony because a queen mates with up to 20 drones (males)(*16*), and thus honey bee colonies typically contain a mixture of many different paternal genotypes. This within-colony genetic diversity of learning capacities may reflect a colony level trait that allows the colony to react and adapt to rapidly changing resource distributions (*17, 18*).

Our objective here was to evaluate the genetic and neural mechanisms that underlie individual differences for latent inhibition in honey bees. We show that a major locus supporting individual differences maps to a location in the honey bee genome previously identified in independent mapping studies as being important for latent inhibition(*19*) as well as for sugar and pollen preferences in foragers (*20, 21*). Disruption of a tyramine receptor encoded by *Amtyr1* in this region changes expression of latent inhibition in a way that suggests that intact signaling via the *Amtyr1* pathway is important for modulating plasticity

at inhibitory synapses. Furthermore, electrophysiological analyses combined with blockade of the AmTYR1 receptor in the antennal lobe – the first synaptic center along the olfactory pathway – decreased antennal lobe responsiveness to odor and blocked a neural correlate of latent inhibition. Finally, sequencing the gene failed to reveal mutations in the coding regions that would affect protein function, leading to the conclusion that variation across workers could arise from differential gene expression through transcriptional regulation. We discuss how these data strongly imply a functional role for *Amtyr1* signaling in modulating expression of attention via latent inhibition. These findings are also important for understanding the strategies colonies use to explore for and exploit pollen and nectar resources(*22*).

Results

We used two genetic lines of honey bees that had been bred for high (inhibitor) or low (noninhibitor) expression of latent inhibition. These lines were independently selected using identical methods to a previous study that had successfully bred high and low lines(*19*). We evaluated 523 recombinant drones generated from a single hybrid queen produced from a cross between a drone from a noninhibitor line and a queen from an inhibitor line (Fig. 1). Honey bee drones are ideal for behavior genetic studies because they are haploid progeny that develop from an unfertilized egg laid by the queen. We then selected 94 high and 94 low performing drones for the Quantitative Trait Locus (QTL) analysis, which identified one significant locus (Fig. 2A). The QTL mapped to the same genomic region identified in a previous study of latent inhibition (called '*lrn1'*) using an independent inhibitor and noninhibitor cross and different (RAPD-based) genetic markers (*19*). This is the same genomic region that has been identified in studies of foraging preferences of honey bees (*21, 23*), where it has been called *pln2* for its effect on pollen versus nectar preferences and in modulating sensitivity to sucrose (*24*). Clearly this genomic region has major effects on several foraging-related behaviors.

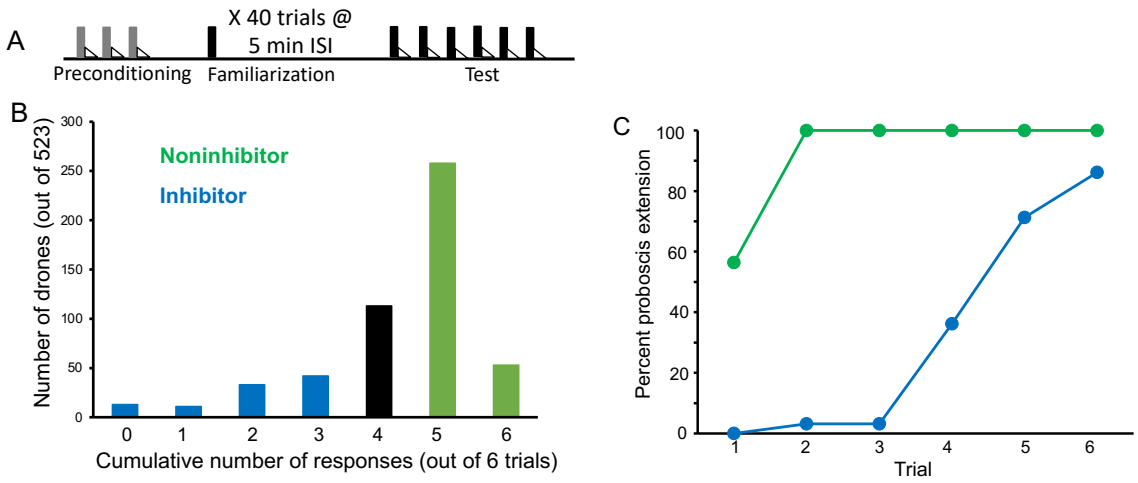
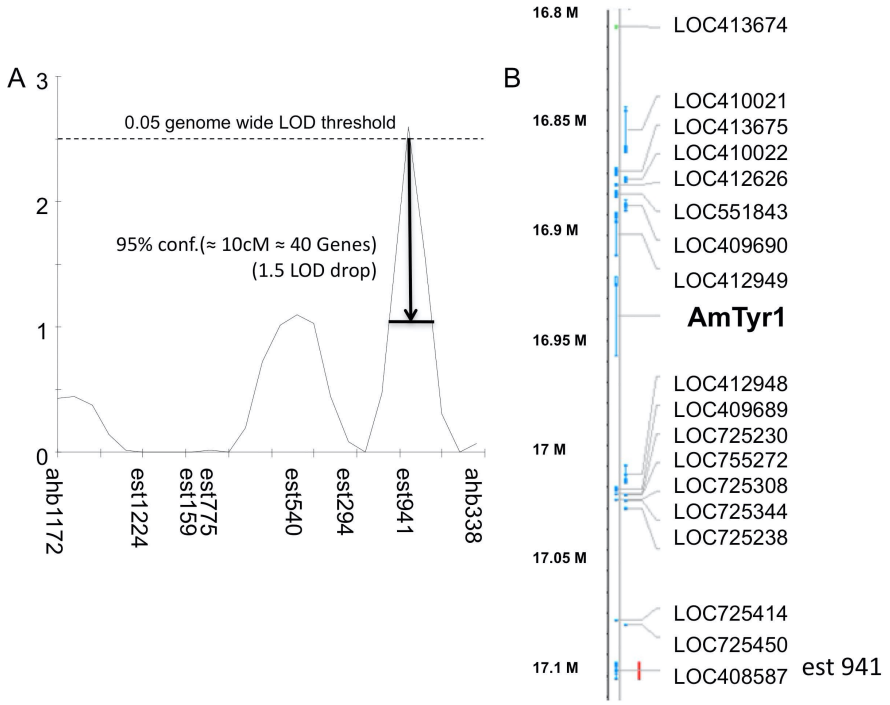


Fig. 1. Evaluation and selection of drones from an F1 queen. **(A)** Drone honey bees were first evaluated by conditioning them over three trials to an odor (A; gray bars) followed by sucrose reinforcement (triangles) in a way that produces robust associative conditioning expressed as odor-induced proboscis extension response (PER) (*25*). All drones that showed no PER response on the first trial and PER response on each of the following two trials were selected for the subsequent familiarization phase. This procedure ensured that only drones motivated to respond to sucrose and learn the association with odor were selected. Approximately 10% of honey bees fail to show evidence of learning in PER conditioning using the collection methods described in Materials and Methods. The familiarization phase involved 40 4-sec exposures to a different odor (X; black bars) using a 5 min interstimulus interval. These conditions are sufficient for generating latent inhibition that lasts for at least 24 h (*6*). Finally, the test phase involved 6 exposures to X followed by sucrose reinforcement. **(B)** Frequency distribution of 523 drones evaluated in the test phase. The x-axis shows the summed number of responses over 6 conditioning trials. Fewer responses correspond to stronger latent inhibition. A total of 94 drones were selected in each tail of the distribution. 'Inhibitor' drones showed zero through 3 responses, and 'Noninhibitor' drones showed 5 or 6 responses. **(C)** Acquisition curves for the 94 inhibitor and noninhibitor drones. Approximately half of the noninhibitor drones showed spontaneous responses on the first trial, which is typical for noninhibitors in latent inhibition studies of honey bees (*6*). All of the drones in that category showed responses on trials 2 through 6. In contrast, inhibitor drones showed delayed acquisition to the now familiar odor.

When we analyzed the gene list within the confidence intervals of this QTL, one gene - *Amtyr1* - in particular stood out (Fig. 2A,B). That gene encodes a biogenic amine

receptor for tyramine (AmTYR1) (*26*) that is expressed in several regions of the honey bee brain (*27-29*). AmTYR1 is most closely related to the insect alpha-2-adrenergic-like octopamine receptors and the vertebrate alpha-2-adrenergic receptors (*30*). Activation of AmTYR1 reduces cAMP levels in neurons that express it. We specifically considered AmTYR1 for more detailed evaluation for several reasons. Tyramine affects sucrose sensitivity in honey bees(*31*), and nurses and foragers differ in AmTYR1 expression(*32*). Mutations in the orthologous tyramine receptor in fruit flies disrupt odor-guided innate behaviors to repellants (*33*). Tyramine is also the direct biosynthetic precursor to octopamine(*34*), which has been widely implicated in sucrose-driven appetitive reinforcement learning in the honey bee (*35, 36*). Therefore, Ventral Unpaired Medial neurons, which lie on the median of the subesophageal ganglion in the honey bee brain (*28, 37-39*), and which form the basis for the appetitive reinforcement pathway must produce tyramine in the process of making octopamine. Recent analyses indicate these neurons in locusts and fruit flies also release both neuromodulators when activated (*40, 41*). Finally, octopamine and tyramine affect locomotor activity in the honey bee(*42*).



142 **Fig. 2. Single-Nucleotide Polymorphism (SNP) mapping of high and low recombinant**
143 **drones. (A)** Markers from linkage group 1.55 surrounding one significant QTL (est941 with a LOD
144 score of 2.6). **(B)** Partial list of genes within 10 cM of the marker showing the location of *Amtyr1*.

145 We then evaluated whether non-synonymous mutations in the coding sequence might
146 change the functionality of the receptor. We performed a detailed genomic analysis of the
147 40kb region including the *Amtyr1* gene, a 2kb upstream, and a 0.5kb downstream non-
148 coding region. SNP frequency in the coding sequence (CDS) was relatively low compared
149 to the genome wide SNP frequency, and all 46 SNPs in the coding regions in any of the
150 sequenced eight individual worker genomes represented synonymous substitutions, i.e.,
151 these SNPs do not change the sequence of the encoded protein. Thus, phenotypic differ-
152 ences are not caused by structural changes in the tyramine receptor protein itself. We did,
153 however, find an increased SNP frequency in introns, the up- and downstream non-coding
154 regions and the 3' untranslated region (UTR). If *Amtyr1* is involved in latent inhibition,
155 these variations might be linked to the changes in the regulation of *Amtyr1* gene expres-
156 sion, e.g., by changes in transcription factor binding sites or the stability of the mRNA,
157 which might eventually be responsible for the observed phenotypic differences.

158 **Disruption of *Amtyr1* affects expression of latent inhibition**

159 To further examine the role of *Amtyr1* signaling in latent inhibition, we performed a
160 series of behavioral experiments that involved treatment of honey bees either with the
161 tyramine receptor antagonist yohimbine (*43*) or with a Dicer-substrate small interfering
162 (Dsi) RNA of the receptor (NCBI Reference Sequence: NM_001011594.1) to disrupt
163 translation of mRNA into AmTYR1 (*28, 44*). For these experiments we used unselected
164 worker honey bees from the same background population used for selection studies,
165 which ensured that workers used for behavioral assays would represent a mixture of in-
166 hibitor and non-inhibitor phenotypes. Therefore, treatment could increase or decrease the
167 mean level of latent inhibition in this population. Training involved two phases (Fig. 3A).
168 First, during the 'familiarization' phase honey bees were identically exposed over 40 trials
169 to odor X without reinforcement. Our previous studies have shown that this procedure
170 produces robust latent inhibition. The second 'test' phase involved measurement of latent
171 inhibition. During this phase odor X and a 'novel' odor N were presented on separate trials.
172 Both odors were associated with sucrose reinforcement in a way that produces robust

appetitive conditioning (*25*). Latent inhibition would be evident if responses to odor X were lower than the responses to the novel odor N. Injections of yohimbine directly into brains occurred either prior to the familiarization phase (Fig. 3A,B) or prior to the test phase (Fig. 3C).

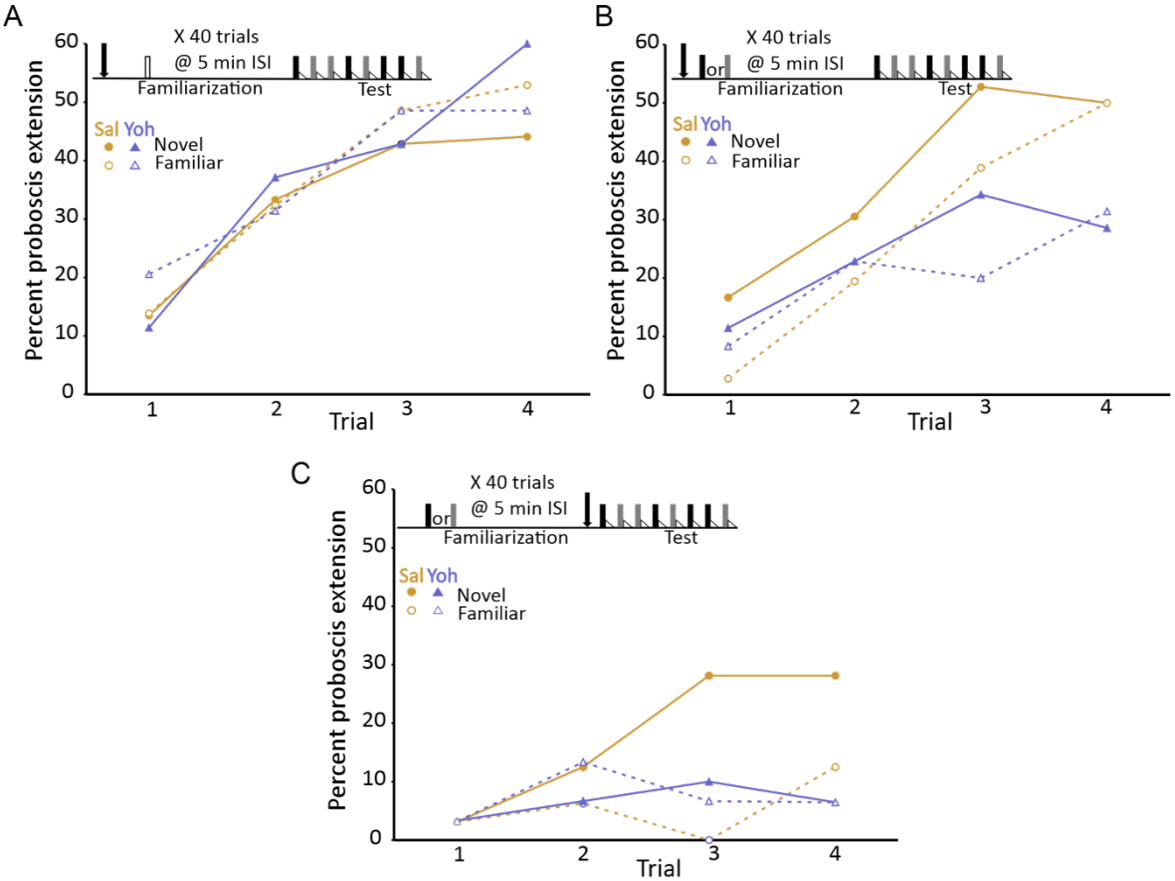


Fig. 3. Blockade of the tyramine receptor with yohimbine modulated expression of latent inhibition. (A) Acquisition during the test phase in two injection groups of honey bees familiarized to air as a control procedure to evaluate the effects of yohimbine on excitatory conditioning. The conditioning protocol is shown at the top. In this experiment (and in Fig 3**B** and **C**) we omitted the first phase (Fig. 1**A**), which does not affect expression of latent inhibition(*6*) and is only necessary when subjects are being selected for development of genetic lines. One group was injected (arrow) with saline (orange circles; n = 37 animals) and the other with yohimbine (blue triangles; n=35) prior to familiarization. Because there was no odor presented during familiarization (open box), odors during the test phase were both 'novel' when conditioned. The test phase in this experiment (also in Fig 3**B** and **C**) differed from the test phase in Figure 1. For this design, each subject was equivalently conditioned to both odors on separate, pseudorandomly interspersed trials. Acquisition to both odors in both injection groups was evident as a significant effect of trial (X^2=47.5, df=3,

190 p<0.001). None of the remaining effects (odor, injection or any of the interaction terms) were sig-
191 nificant (p>0.05). **(B)** As in **A**, except both groups (orange saline: n=36; blue yohimbine: n=36) in
192 this experiment were familiarized to odor; each odor (gray and black boxes; see Methods) was
193 familiarized in approximately half of the animals in each injection group. In this design, each indi-
194 vidual was equivalently conditioned to both odors during the test phase; latent inhibition is evident
195 when the response to the novel odor is greater than to the familiar odor. Injection was prior to odor
196 familiarization. **(C)** As in **B**, except injection of saline (n=32) or yohimbine (n=30) occurred prior to
197 the test phase. Statistical analysis of data sets in **B** and **C** yielded a significant interaction (X^2=7.4,
198 df=1, p <0.01) between injection (saline vs yohimbine) and odor (novel vs familiar) that was the
199 same in both experiments, as judged by the lack of a significant odor*injection*experiment inter-
200 action term (p>0.05). There was a higher response to the novel odor than to the familiar odor, but
201 only in the saline injected groups. The lower rate of acquisition in **C** (X^2=64.0,1,p<0.01) could be
202 due to performance of this experiment at a different time of year, or to injections immediately prior
203 to testing, which affects levels PER conditioning in honey bees but leaves intact relative differ-
204 ences between groups (*45*).

205

206 The first experiment provided an important control procedure to evaluate whether yo-
207 himbine affects excitatory conditioning. This procedure involved familiarization to air,
208 which does not induce latent inhibition to odor (*6*). Honey bees familiarized to air learned
209 the association of both odors with sucrose reinforcement equally well (Fig. 3A). The re-
210 sponse to each odor significantly increased, as expected, across trials (X^2=47.5, df=3,
211 p<0.001). Moreover, there was no effect of injection with saline versus yohimbine; the re-
212 sponse levels to all four odors across the saline and yohimbine injection groups were
213 equivalent. Therefore, blockade of tyramine signaling does not affect excitatory condition-
214 ing, which is an important control for the effects about to be described. This control pro-
215 cedure also shows that yohimbine at 10^{-4} M probably does not affect receptors for other
216 biogenic amines, such as octopamine, dopamine and serotonin, all of which have been
217 shown to have specific effects on appetitive olfactory learning in honey bees (*35, 46-49*).

218 Yohimbine treatment affected the expression of latent inhibition in both treatments that
219 involved familiarization to odor (the interaction between novel vs familiar odor and saline vs
220 yohimbine injection: X^2=7.4, df=1, p <0.01). First, in the saline controls, honey bees re-
221 sponded more often to odor N than to X after injection of saline prior to familiarization or

222 prior to testing (Fig. 3B,C circles). The response to the familiar odor was lower than the
223 response to the novel odor on most trials, including spontaneous responses on the first
224 trial. Injection of yohimbine eliminated the difference in response to the novel and familiar
225 odors. Moreover, the responses to both odors after yohimbine treatment were significantly
226 lower than, or at least equal to, the response to the familiar odor in the respective saline
227 controls. This pattern could not arise from blockade of excitatory learning about N, be-
228 cause excitatory learning was unaffected in the air preexposure controls (Fig 3A). Instead,
229 the yohimbine-induced pattern was specific to the treatments in which one odor was fa-
230 miliar.

231 This result implies that blockade of AmTYR1 modulates latent inhibition to a familiar
232 odor and that the effect now generalizes to the novel odor. Finally, the relative effect of
233 yohimbine treatment, i.e. reduction of PER response rate, is similar when it is injected
234 either prior to familiarization (Fig. 3B) or prior to testing (Fig. 3C). This pattern, that is, the
235 same effect prior to acquisition or testing, is similar to the action of octopamine blockade
236 on excitatory conditioning (*35*).

237 Although the results with yohimbine were promising, we were concerned that yohim-
238 bine can have effects on other receptors, specifically on an α2-adrenergic-like octopamine
239 receptor (*30*) and on an excitatory tyramine receptor AmTYR2*(43).* Therefore, we decided
240 to disrupt *Amtyr1* expression via injection of *Amtyr1* DsiRNA in order to provide an inde-
241 pendent method to test the role of AmTYR1 in producing latent inhibition (Fig. 4). Yohim-
242 bine blocks the receptor, whereas dsiAmtyr1 disrupts production of the receptor protein.
243 Similar outcomes with the two different methods would increase confidence in the result.
244 For the behavioral experiments we used the same procedure as above for yohimbine
245 except that the mixture of three *Amtyr1* DsiRNA constructs was injected 20 hours prior to
246 conditioning because of the time frame needed for the DsiRNA to target mRNA. Because
247 of that time frame, and because injection of yohimbine prior to either phase produced
248 equivalent results, we performed injections of *Amtyr1* DsiRNA only prior to familiarization.
249 As a control we used a scrambled sequence of *Amtyr1* (DsiScr). Use of DsiScr controls
250 for possible nonspecific effects arising from any aspect of the injection.

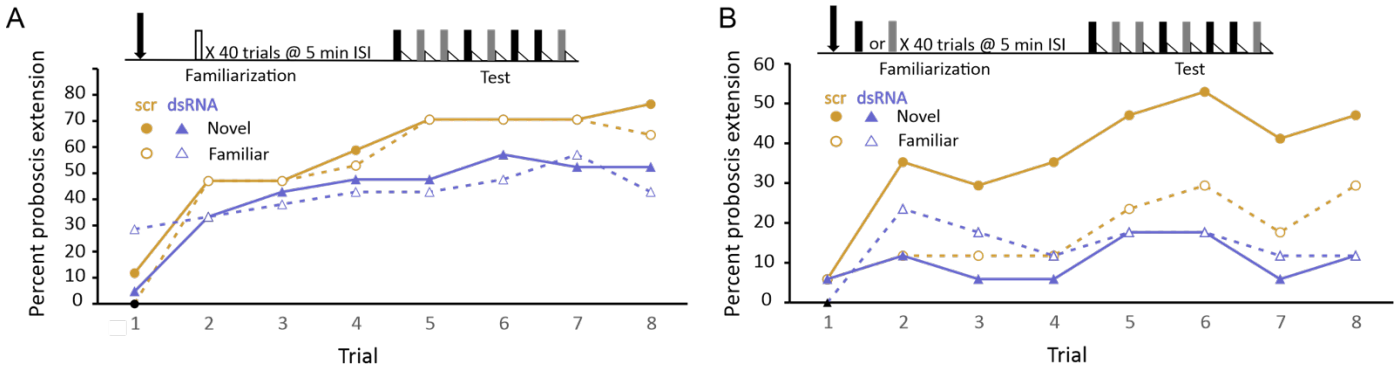


Fig. 4 Disruption of translation of the tyramine receptor by DsiRNA also modulated expression of latent inhibition. This experiment was identical to that shown in Fig 3A,B, except injections were performed with a mixture of Dsi *Amtyr1*RNA (dsRNA; arrow) 24 hour prior to behavioral training and testing. The control for this experiment was a scrambled sequence of the *Amtyr1* RNA, Dsiscr (scr). **(A)** After treatment with Dsiscr (N=17) or DsiRNA (N-19) and familiarization to air, acquisition to both odors was significant across trials (X^2=62.7,7,p<<0.01). There was also a significant effect of injection (X^2=8.8, 1, p<0.01). However, the odor*injection interaction was not significant. **(B)** Same as in A, except familiarization was to odor (Dsiscr (N=17) DsiRNA (N=13)). The injection*odor interaction was significant (X^2=7.8; 1; p<0.01. Quantitative PCR analysis of *Amtyr1* mRNA levels in brains revealed lower levels of mRNA in Dsi*Amtyr1* injected animals (0.046+/-0.006) than in Dsiscr injected animals (0.142+/0.028).

Injection of *Amtyr1* DsiRNA produced the same effects as yohimbine. After familiarization to air as a control, both groups of foragers learned the association of both odors (X^2=62.7, 7, p<<0.01; Fig. 4A), although there was a slight decrement in response rate in DsiRNA injected animals (X^2=62.7, 7, p<<0.01; see discussion below). In contrast, after familiarization to one of the odors, learning of both the novel and familiar odors was poor in the *Amtyr1* DsiRNA injected group (Fig. 4B). But expression of latent inhibition was normal – i.e. responses to the novel odor exceeded the responses to the familiar odor in the DsiScr group. As before the interaction between odor and injection was significant (X^2=7.8, 1, p<0.01).

In conclusion, both behavioral experiments support the hypothesis that *Amtyr1* affects expression of latent inhibition without affecting excitatory conditioning. However, the results at first glance seemed counterintuitive. Blockade and disruption of *Amtyr1* did not

276 attenuate latent inhibition by, for example, increasing the responsiveness to the familiar
277 odor. Instead, treatment with yohimbine or *Amtyr1* DsiRNA reduced responsiveness to
278 the novel odor. This result is consistent with *Amtyr1* modulating inhibition involved in, for
279 example, identified inhibitory processes in the antennal lobes and or the CNS(*50*). Spe-
280 cifically, and as we propose below in a model, it would prevent the inhibition from becom-
281 ing too strong, and possibly keep it at a set point between very strong and very weak.

282 **Disruption of *Amtyr1* signaling affects neural codes for odors in the antennal lobe**

283 Because of this intriguing result, we performed additional experiments to investigate
284 the mechanism in more detail. Our prior studies of odor coding identified neural manifes-
285 tations of latent inhibition in early synaptic processing of the antennal lobes of the honey
286 bee brain(*51, 52*). Familiarization to an odor X caused a mixture of a novel odor N and X
287 to become much more like N(*52*). That is, neural information about familiar odors like X is
288 filtered out of mixtures. Furthermore, responses to any novel odor are enhanced after
289 familiarization to X(*51*), which is a form of novelty detection. These effects in the antennal
290 lobes could arise because of expression of AmTYR1 in presynaptic terminals of sensory
291 axons in the honey bee antennal lobes(*28*), where activation of AmTYR1 would decrease
292 cAMP levels(*26*) and likely decrease release of acetylcholine at synapses.

293 We therefore chose to analyze the effect of yohimbine treatment on odor processing in
294 the antennal lobes by recording electrophysiological responses to odors prior to and after
295 familiarization in combination with yohimbine treatment. We used yohimbine in these ex-
296 periments because of the more rapid onset (minutes versus hours) compared to DsiRNA
297 treatment. This first experiment did not employ familiarization to odor. Prior to yohimbine
298 treatment, recordings from 71 units across 4 animals revealed responses to odors that
299 ranged from no detectable change in spike activity with odor presentation to a robust in-
300 crease in spiking activity (Fig.5A). After yohimbine treatment, responses decreased pre-
301 cipitously, although spiking activity was still detectable (Fig. 5B). This decrease in re-
302 sponse is consistent with AmTYR1 being involved in regulation of inhibition in networks
303 of the antennal lobe, which are the first order processing centers for odors. Hypothetically
304 at least, when AmTYR1 is blocked by yohimbine, excitation of inhibitory networks in the
305 antennal lobe increases and drives down PN responses.

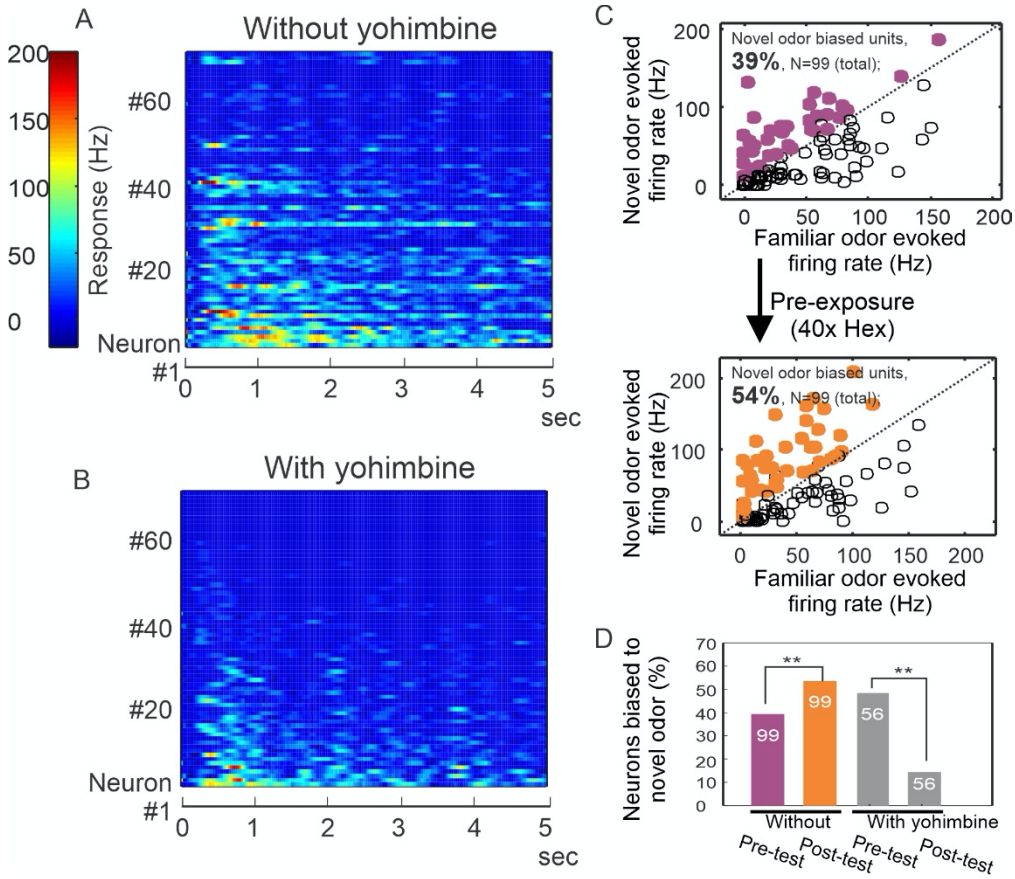


Fig. 5 Yohimbine disrupts processing in the honeybee antennal lobe. **A-B**. Perfusion of yohimbine solution (50μM in physiological saline) into honey bee head capsule caused antennal lobe units to decrease response magnitude to odor stimuli (2-octanone and 1-hexanol) in general. Odor was delivered through a solenoid valve that was open at time zero and lasted for 4 secs. **C**. In control experiments where yohimbine was not applied, most of the units were responsive to both hexanol and octanone, but 39% were biased towards octanone (purple dots above the diagonal line), i.e. showing stronger response to octanone than to hexanol. During the familiarization protocol, these units were familiarized to hexanol 40 times with 1 min interval (arrow down) and were tested again with hexanol and octanone 10 min after the last odor stimulation in the familiarization phase. The test results show 54% of units responded more strongly to octanone (orange dots), which is a novel odor in this protocol. The 15% increase is statistically significant (*McNemar* test with Yates's correction, d.f.=1, Chi-square=5.939, $p<0.02$) (asterisks on purple and orange bars, N=99). **D**. When the familiarization protocol was used with saline versus yohimbine perfusion, the response bias towards novel odor was disrupted, showing a significant decrease in comparison with the familiar odor (*McNemar* test with Yates's correction, d.f.=1, Chi-square=11.13, $p<0.001$) (asterisks on grey bars, N=56).

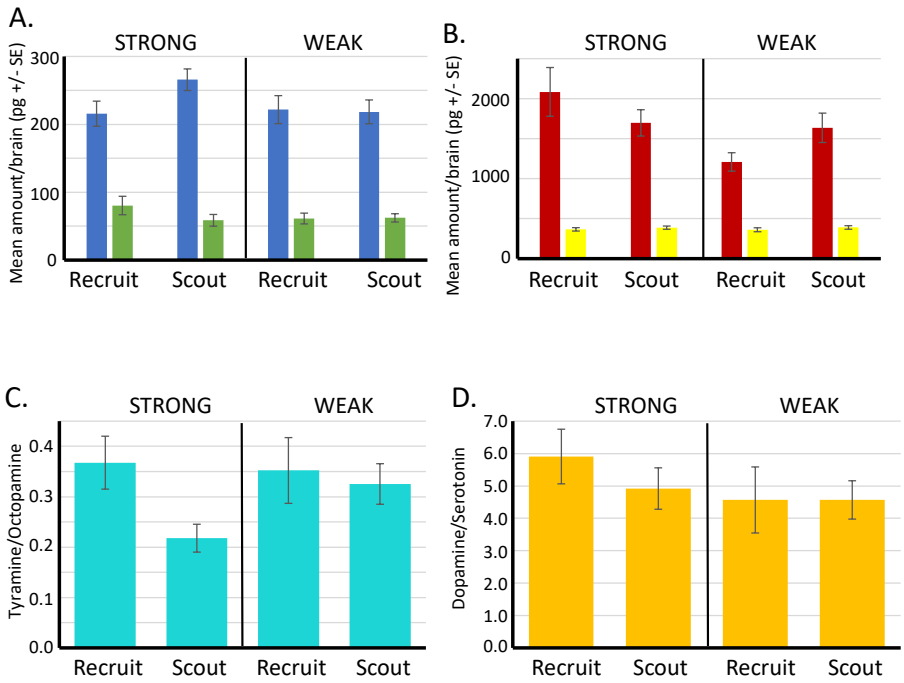
323

324 We then evaluated whether continuous perfusion of the brain with saline or yohimbine
325 *during odor familiarization* would interrupt how latent inhibition is manifested in the anten-
326 nal lobe by potentiation of responses to novel odors, as we have reported(*51*). Indeed,
327 yohimbine treatment modified how neurons respond to novelty. Using the same familiari-
328 zation protocol as in Figs 3 and 4, but under conditions of saline perfusion, we found that
329 39% of units (N=99) responded more strongly to the Novel odor before the familiarization
330 to an odor (Fig.5C, purple dots in upper panel; purple bar in Fig.5D). After familiarization,
331 this percentage increased significantly to 54% (Fig.5C, orange dots in lower panel; orange
332 bar in Fig.5D) (*McNemar* test with Yates's correction, d.f.=1, Chi-square=5.939, $p<0.02$).
333 Hence familiarization increased bias towards the novel odor in neurons that were more
334 responsive to that odor to begin with, which is consistent with our earlier results(*51*). In
335 different experiments where yohimbine was perfused, the familiarization protocol not only
336 did not increase bias towards the Novel odor, it significantly decreased the original bias
337 from 49% (N=56) to 14% (Fig.5D, grey bars) (*McNemar* test with Yates's correction,
338 d.f.=1, Chi-square=11.13, $p<0.001$), suggesting that yohimbine interrupted this neural
339 manifestation of latent inhibition in the antennal lobes.

340 **The tyramine/octopamine ratio in the brain is also associated with latent inhibition**

341 A recent report implicated the release of dopamine in driving reward seeking behavior
342 (*53*). In order to evaluate whether dopamine might be involved in latent inhibition, and
343 whether change in release of octopamine and/or tyramine might contribute to our behav-
344 ioral results, we reanalyzed previously published data (*54*) on levels of dopamine, seroto-
345 nin, octopamine and tyramine in individual brains of 81 foragers collected from an unse-
346 lected genetic background used for selection of lines for expression of latent inhibition.
347 The foragers were collected as 'scouts' or 'recruits'. Scouts were defined as the first bees
348 to explore a new landscape into which their colony had been moved. Recruits were de-
349 fined as foragers that were exploiting resources once they were found. All scouts and
350 recruits were trained for latent inhibition in the laboratory, and then classified as to whether
351 they showed strong or weak latent inhibition based on learning a novel and familiar
352 odor(*54*).

353 Of the biogenic amines (Figs. 6A,B), only tyramine showed differences between scouts
354 and recruits (see (*54*) for methods and a more complete analysis of these data). Differ-
355 ences in dopamine or serotonin levels were not significant. For the current purpose, we
356 re-analyzed the data to focus on the ratios of tyramine to octopamine and dopamine to
357 serotonin (serotonin was used a reference for dopamine levels in (*53*)). Scouts that
358 showed strong latent inhibition also had significantly lower ratio of tyramine to octopamine
359 than recruits, and that ratio was also lower than scouts and recruits that showed weak
360 latent inhibition (Figs. 6C,D). There were no significant differences in the dopamine to
361 serotonin ratios. Thus, there is an interaction of tyramine and octopamine *production* with
362 behavioral division of foraging labor and expression of latent inhibition. However, dopa-
363 mine, serotonin and their ratios do not appear to be involved in latent inhibition.



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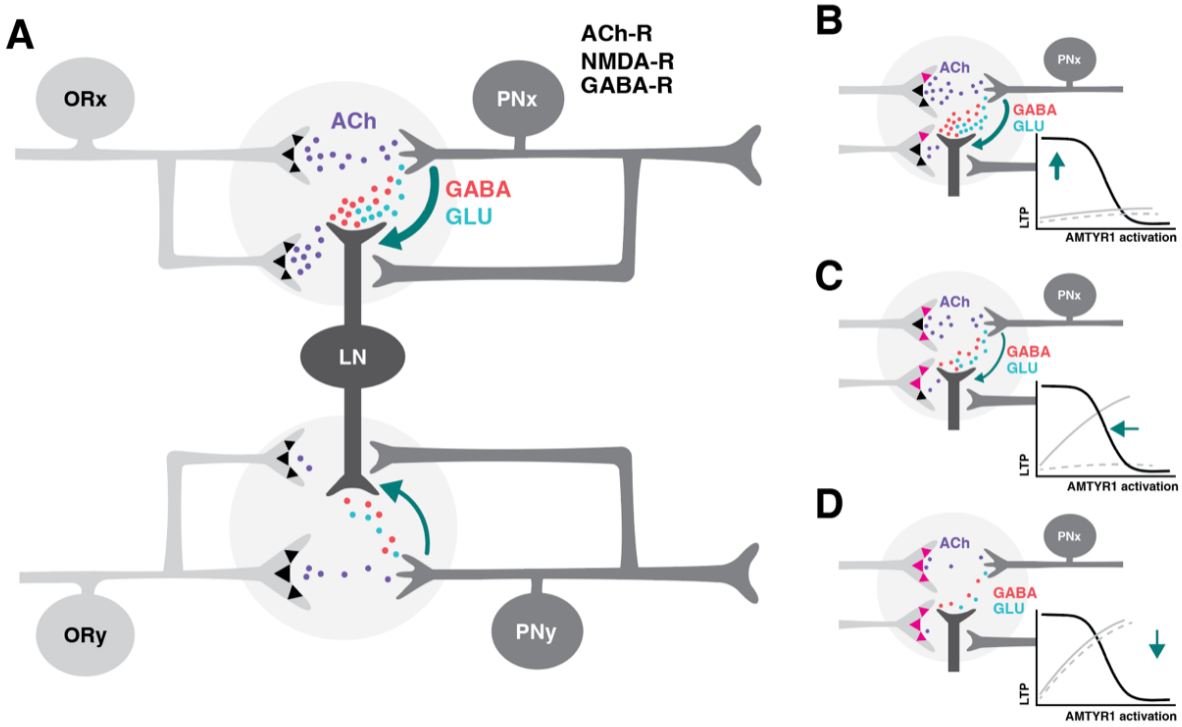
365 **Fig. 6. Biogenic amine levels in individual brains of scout and recruit foragers that ex-**
366 **pressed strong or weak latent inhibition. A-B.** Absolute levels of octopamine (blue) and tyra-
367 mine (green) and of dopamine (red) and serotonin (yellow) in individual forager brains. **C.** Ratios
368 of tyramine/octopamine. In foragers that exhibited strong latent inhibition, the ratio was signifi-
369 cantly lower in scouts (N=25) than recruits (N=13) (Wilcoxon W=56.0, $p<0.05$). Ratios did not differ
370 in scouts (N=24) and recruits (N=19) that exhibited weak latent inhibition ($p>0.05$) **D**. Ratios of

371 dopamine/serotonin did not differ in either the strong or weak groups (p>0.05). Sample sizes the
372 same as in C.
373
374 ## Discussion
375 Our results have identified the genetic and neural underpinnings that modulate an im-
376 portant form of learning and memory in the brain. All animals need to learn about stimuli
377 in their environment. Latent inhibition is important for redirecting limited attention capacity
378 away from unimportant, inconsequential stimuli and refocusing it toward novel stimuli
379 about which the animal knows little or nothing. Two independent QTL mapping studies
380 have now identified the genetic locus that contains *Amtyr1* as important for regulating
381 individual variation in attention(*19*). There are other loci in the genome that show associ-
382 ations with the behavior, and there are also other unidentified genes in the same locus.
383 Nevertheless, our manipulation of *Amtyr1* function using both pharmacology and DsiRNA
384 treatments confirm its association with behavioral expression of latent inhibition.

385 There are a few points that need to be kept in focus at this point in our understanding
386 of *Amtyr1*. First, it is not a *latent inhibition gene*. Instead, it is a gene that has broad effects
387 on foraging-related behaviors that include its effect on expression of latent inhibition. In
388 that sense it has major effects on a broader behavioral syndrome that includes effects on
389 sucrose sensitivity (*24, 29, 31*), preferences for nectar and/or pollen (*21*), behavioral caste
390 differences (*32*), reproductive physiology (*55*) and learning (*12*). It remains to be deter-
391 mined how *Amtyr1* can have such broad effects. Part of the answer undoubtedly lies in its
392 expression in several areas of the brain (*28*). Additionally, as we discuss below, it is likely
393 modulating afferent processing that, in the case we describe here, drives Hebbian plas-
394 ticity.

395 Second, the effects of *Amtyr1* specifically on expression of latent inhibition likely arise
396 by combining its expression in sensory as well as more central areas of the brain. That is,
397 it is unlikely that there is a single locus in the brain that underlies latent inhibition. We have
398 shown for honey bees(*28*), for example, that AmTYR1 is on presynaptic terminals of Ol-
399 factory Sensory Neuron axons in the antennal lobes, where they provide excitation to
400 dendrites of Local GABAergic Inhibitory Interneurons (LN) and Projection Neurons (PN)
401 (Fig. 7A). AmTYR1 is also on presynaptic terminals of Projection Neuron axons that

402 terminate in the mushroom body calyces. In the antennal lobe, where our electrophysio-
403 logical and imaging studies have focused (*51, 52, 56, 57*), familiarization to an odor
404 causes the neural representation of a mixture that contains the familiar odor to become
405 more like novel odors in the mixture (*52*). And it potentiates the response to the novel
406 odor(*51*). We assume, but have yet to show experimentally, that this bias combines with
407 how AmTYR1 affects processing in the mushroom bodies, where olfactory information
408 converges with information from other sensory modalities. These higher-order effects of
409 AmTYR1 could underlie why individuals from genetic lines selected in the laboratory for
410 odor-based latent inhibition show differential attention to different sensory stimuli associ-
411 ated with feeders when tested in free flying conditions in the field (*22*).



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413 **Figure 7. Hypothetical model of how *Amtyr1* could modulate hebbian plasticity in feed-for-**
414 **ward and lateral inhibition to modulate latent inhibition.** A. Circuitry of two glomeruli (Figure
415 7 A has been adapted from Figure P1 from (*58*)) that underlies latent inhibition (habituation) in the
416 fruit fly antennal lobe, and which was also proposed via a computational model to underlie
417 changes in antennal lobe responses to familiar and novel odors in the honey bee (*52*). In the fruit
418 fly(*58*), ORNs release Ach (purple) to coactivate PNs and LNs, which both express ACh receptors.
419 LNs release both GABA (red) and glutamate (green) onto synapses with PNs. NMDA receptors

420 on PNs potentiate the LN/PN synapses via a retrograde signal (green arrows) to LNs. This hebbian
421 plasticity increases inhibition of PNs by LNs and thus decreases excitation of the PN. LN/PN co-
422 activation in other glomeruli is either non-existent (e.g.(*58*)), too low to produce potentiation of the
423 LN/PN synapse, or strong enough to produce inhibition to odors other than the familiar odor.
424 AmTYR1 receptors on presynaptic terminals of ORNs (*28*) are shown as black triangles. Here we
425 do not show the source for tyramine. We assume it could be from the VUM neuron, which has
426 terminals in the cortex of every glomerulus, where ORN terminals overlap with PN and LN den-
427 drites(*59*). B-D. Increasing activation of AmTYR1 (pink triangles) progressively decreases release
428 of ACh and lowers coactivation of LNs and PNs. Decreased release of glutamate reaches a
429 threshold (D) below which it fails to modify the LN/PN synapse via a decreased retrograde signal,
430 although ACh release might still activate the glomeruli. Graphs inset in each figure show activation
431 of AmTYR1 (x-axis) verses LTP-based hebbian plasticity (y-axis). Low activation of AmTYR1 pro-
432 duces high coactivation-based LTP (B) and vice-versa (D). Arrows show the hypothetical relation-
433 ship in each figure. Gray lines represent the predicted acquisition levels to novel (solid) and famil-
434 iar (dashed) odors given the strength of Hebbian plasticity in each figure. For these lines the x-
435 axis would be 'Trial' and y-axis 'Percent proboscis extension', as in Figs. 3 and 4.

436

437 The precise relationship of *Amtyr1* to latent inhibition is different from what is normally
438 expected from disruption of a gene that underlies a behavior. We expected that disruption
439 of *Amtyr1* function would reduce or eliminate latent inhibition; that is, learning about a
440 familiar odor (X) would rise to equal learning about the novel odor. Instead, the response
441 to the novel odor was reduced to equal that to the familiar odor. This reduction was specific
442 to familiarization treatment, so it is dependent on plasticity. It cannot be explained by non-
443 specific – e.g. toxic – effects of treatment, because the same treatments did not reduce
444 excitatory conditioning in the absence of familiarization to an odor. Moreover, the same
445 effect was evident using two very different means for disruption of *Amtyr1* signaling.

446 We propose that *Amtyr1* modulates neural plasticity that reduces attention to a familiar
447 odor. *Amtyr1* maintains coactivation of LNs and PNs in the antennal lobe at a set point
448 between the extremes where it becomes too strong (e.g. when *Amtyr1* is disrupted) or too
449 weak (*Amtyr1* strongly activated). Given that activation of *Amtyr1* reduces cAMP levels, it
450 would be expected that this would reduce excitability of axon terminals. Hypothetically
451 then, activation of *Amtyr1* could reduce excitatory drive of post-synaptic processes on

452 PNs and LNs in, for example, the antennal lobes, and possibly between PN axons and
453 intrinsic neurons of the mushroom bodies(*28*).

454 **A model of how *Amtyr1* could modulate inhibition in antennal lobe networks**

455 We can now propose a model for how *Amtyr1* could act in the antennal lobes, and
456 possibly also as an important component for encoding latent inhibition in distributed net-
457 works in the mushroom body. The model would have to explain why air (mechanosensory)
458 stimulation alone does not reduce subsequent acquisition to odors (Figs. 3A, 4A) as much
459 as familiarization to an odor (Figs. 3B,C, 4B). Mechanosensory stimulation produces fast,
460 transient responses in antennal lobe glomeruli, whereas odor stimulation produces more
461 robust, longer lasting responses(*60, 61*). These differences in response could underlie the
462 difference in response to air versus odor in our analyses, particularly in driving activity
463 dependent plasticity at LN-to-PN synapses that we describe below.

464 We propose that effects *specific to odor familiarization* occur by amplifying hebbian
465 plasticity between PNs and LNs in the antennal lobe networks (Fig. 7A). A computational
466 model of the honey bee antennal lobe identified hebbian plasticity at Local GABAergic
467 Inhibitory Interneuron (LN) synapses onto Projection Neurons (PN) as the most likely lo-
468 cus of plasticity to give rise to the observed biasing of an odor mixture to be less like a
469 familiar odor and more like a novel odor (*52*). If ORy in Fig 7A is not activated by an odor
470 that activates ORx, then after familiarization to X a novel odor that activates ORy would
471 be better able to suppress X in a mixture because of strengthened LN-to-PNx synapses
472 (see Fig 7 in (*52*)). A potentiated novel odor would strengthen this effect. Work with fruit
473 flies also showed that glutamate/NMDA receptor dependent hebbian plasticity at the same
474 synapses underlies odor habituation ((*58*); the same process as latent inhibition just dif-
475 ferently named).

476 In our model (Fig. 7A-D), coactivation of PNs and LNs via excitation from OR axon
477 terminals would produce plasticity at the LN-to-PN synapses. Low activation of AmTYR1
478 (Fig. 7B) would lead to strong input from sensory axon terminals that would maximally
479 activate LNs and PNs, thus leading to strong Hebbian plasticity at LN-to-PN synapses.
480 That is, it would lead to the strong inhibition of PNs in our manipulations that blocked or
481 disrupted AmTYR1 signaling. At moderate levels (Fig. 7C) this plasticity would give rise

482 to normal latent inhibition in our high lines just as it does in fruit flies. Strong activation of
483 AmTYR1 would weaken the activity and reduce or prevent plasticity (Fig. 7D), and hence
484 lead to learning about both the novel and familiar odors (such as in our genetic lines that
485 do not show strong latent inhibition; (*12*)). How well this model works might depend on a
486 nonlinear thresholding function for coactivation that drives hebbian plasticity (AmTYR1-
487 LTP relationships and expected learning curves shown in Figs. 7B-D).

488 Furthermore, strong activation of lateral projections of the inhibitory interneuron (Fig.
489 7A) might cause generalization of latent inhibition to novel odors, as we have observed
490 with disruption of AmTYR1 signaling. Many of the monomolecular odorants that have
491 been used to study olfaction and olfactory learning in honey bees have neural activity
492 patterns that partially overlap (*62*), such as with the representations for hexanol and 2-
493 octanone. For each odor, PNs in a few of the 160+ glomeruli of the antennal lobe are
494 highly activated, and a subset of other PNs are activated to a lesser degree. Hypotheti-
495 cally at least, if the familiar odor activates some of the ORs from glomeruli that also code
496 for the novel odor, then coactivation of those PNs with lateral inhibition could cause
497 Hebbian plasticity at those synapses too. Under normal circumstances the excitation of
498 those ORs might be too low to potentiate inhibition (Fig. 7C,D). But when AmTYR1 sig-
499 naling is too low or disrupted (Fig 7B), coactivation would increase enough to drive the
500 plasticity and reduce responses to novel odors.

501 We have linked expression of latent inhibition to hebbian plasticity in synapses from
502 inhibitory LNs to PNs in the antennal lobe. Although any behavioral phenomenon likely
503 arises from distributed neural networks in the brain, we focused on the effect of familiari-
504 zation in the antennal lobes because of our prior work analyzing odor processing and
505 latent inhibition there in the honey bee (*52*), and because of reports of latent inhibition in
506 the same networks in the fruit fly (*58*). In particular, we report neural effects (suppression
507 of potentiation to the novel odor) that are produced by the same familiarization treatment
508 that produces behavioral latent inhibition, and like the behavior is affected by blockade or
509 disruption of AmTYR1. We do not specifically identify the type of LN represented in Fig.
510 7, but it could be likely that it would belong to the group of heteroLNs(*63*), which receive
511 excitatory inputs in one glomerulus and transmit inhibition to other glomeruli. Computa-
512 tional modeling suggests that the interglomerular connectivity of heteroLNs should be

513 based on ‘functional networks’ defined by overlapping glomerular activity patterns to sim-
514 ilar odors(*50*). However, Fig 7 only represents a minimal part of the network that we feel
515 is needed to convey how it hypothetically modulates Hebbian plasticity. It will be useful to
516 consider the broader network as represented in Sinakevitch et al (*28*) to more clearly un-
517 derstand how AmTYR1 functions in the antennal lobe, and how it may function in the
518 calyces of the mushroom bodies as well.

519 We show in Figure 5 that treatment with yohimbine to block AmTYR1 reduced re-
520 sponses to odor in general, which is consistent with increased excitation from ORN axon
521 terminals driving inhibition, as represented in Fig 7. In fact, this reduction (in the antennal
522 lobe and possibly the mushroom bodies) could be the reason for slightly lower acquisition
523 in Fig 4A under dsRNA treatment. If true, the reduction in unit responsiveness did not
524 completely block excitatory conditioning to odors.

525 We show the effect of odor familiarization in the antennal lobe as potentiation of the
526 response to a novel odor, which is consistent with our earlier report(*51*). AmTYR1 block
527 coupled to familiarization decreased potentiation of responses to the novel odor relative
528 to the increase normally observed in controls. Although we show that potentiation after
529 familiarization depends on activation state of AmTYR1, we do not, and at this point for
530 lack of data we cannot, represent in Figure 7 what gives rise to it. Potentiation could occur
531 via an as yet unknown mechanism intrinsic to the antennal lobe neural networks(*28*). Al-
532 ternatively, it could occur via identified feedback pathways to the antennal lobe (*64, 65*)
533 from neural mechanisms in the mushroom bodies that are known to produce potentiation
534 to novel stimuli in fruit flies(*66*). Under normal circumstances AmTYR1 is functional and
535 moderating the Hebbian plasticity at LN-to-PN synapses at levels consistent with Figs 7C
536 and 7D, where novel odors are learned well (likely aided by potentiation). Blocking or
537 disrupting AmTYR1 puts the network in a state consistent with Fig 7B, where responses
538 to both types of odors are affected by Hebbian plasticity at LN-to-PN synapses – including
539 potentiated novel odors given the overlap in sensory representations.

540 Finally, it remains to be determined what the source for tyramine could be, and which
541 of the local interneuron types might be the ones mediating latent inhibition in the antennal
542 lobes. VUM neurons are an obvious possibility for the source of tyramine, since tyramine

543 is a direct precursor to octopamine released by VUM neurons(*67*). However, this hypoth-
544 esis would depend on tyramine being released at almost constant, low levels without stim-
545 ulation of VUM by taste receptors sensitive to sugars.

546 **Ideas and Speculation**

547 The modulatory role for inhibition that we propose for *Amtyr1* could help to explain its
548 broad involvement in many different behaviors. Differing degrees of *Amtyr1* activation in
549 different neural circuits in the central or peripheral nervous system might regulate activity
550 in those circuits to drive behaviors in one direction or the other; for example, toward high
551 or low sensitivities to sucrose(*31*), preferences for pollen versus nectar(*23*), and states of
552 worker reproductive physiology(*55*).

553 We were initially drawn to *Amtyr1* because of its relationship potentially to the release
554 of tyramine by identified VUM neurons, which have been implicated in excitatory condi-
555 tioning through release of octopamine (*35, 36*). VUM neurons must make tyramine in the
556 process of making octopamine, and they likely release both biogenic amines. In particular,
557 the dynamic balance between octopamine and tyramine is important for regulating insect
558 behaviors (*40, 41*). It is intriguing to now propose and eventually test whether a balance
559 between octopamine and tyramine release from VUM neurons is critical for driving atten-
560 tion in one direction or another depending on association with reinforcing contexts. In this
561 model, activation of VUM neurons would release octopamine to drive excitatory associa-
562 tion between odor and reinforcement. At the same time, release of tyramine would sup-
563 press excitatory drive onto inhibition. Both processes could synergistically drive the asso-
564 ciation. Furthermore, if there is a low level of background tyramine release from VUM
565 when unstimulated, it would explain why in the *Amtyr1* DsiRNA injected group in Fig 4A
566 responded slightly lower than the Dsiscr control group.

567 Interestingly, we have identified a potential interaction in the ratio of tyramine to octo-
568 pamine between foraging role (scouts versus recruits) and expression of latent inhibition.
569 The lower tyramine-to-octopamine ratios in scouts would potentially activate this receptor
570 even less that it would normally be, yielding stronger inhibition according to the model
571 described above. Further analyses need to test this prediction in more detail and evaluate
572 its role in the foraging ecology of honey bees.

Finally, why do individuals in colonies under quasi-natural conditions differ in expression of latent inhibition, and presumably in the functioning of *Amtyr1*? We have used this naturally occurring and selectable genetic variation to establish colonies composed of different mixtures of genotypes(*22, 68*). The mixture of genotypes in the colony affects whether and how quickly colonies discover new resources via an attention-like process operant in individual foragers(*68*). We have therefore proposed that genetic variation leading to colony level variation in *Amtyr1* expression represents a balance between exploration for and exploitation of resources. The precise balance of genotypes would give colonies flexibility to respond to changing resource distributions over the life of the colony.

Box 1. Important questions that need to be addressed in honey bees and other animal models, such as the fruit fly, and in computational models:

- To what extent is tyramine constantly released at a background level, such that it modulates activity of AmTYR1?
- What is the balance of octopamine and tyramine during odor stimulation and when the odor is associated with reinforcement?
- How does the action of AmTYR1 in distributed networks, such as the antennal lobes and mushroom bodies, coordinate to produce behavioral expression of latent inhibition (Fig. 7)?
- How is *amtyr1* activity regulated by other genes in a network, and by epigenetic factors in the environment?

Materials and Methods

Selection of honey bee lines for differences in latent inhibition. We established high and low latent-inhibition lines by conditioning drone and virgin queen honey bees to odors in three different conditioning phases(*12*). The first phase involved selection of drones or queens that could successfully learn to associate an odor with sucrose reinforcement, which established that the honey bees were motivated to learn. This initial excitatory conditioning does not affect generation or expression of latent inhibition. The second 'familiarization' phase involved 40 unreinforced odor exposures for 4 sec each; this new odor (black box; X) was different and discriminable from the first odor. The third and final phase involved conditioning honey bees to the now familiar odor X associated with sucrose

604 reinforcement in a way that normally produces robust associative conditioning (*25*). Strong
605 latent inhibition should slow the rate of learning to X. Drones and queens that exhibited
606 this 'inhibitor' phenotype (defined as zero or one response to X over six conditioning trials)
607 (*12*) were mated using standard instrumental insemination techniques (*69*) for honey bees
608 to create a high (inhibitor) latent inhibition line. Drones and queens that learned X quickly
609 (five positive responses to X over six trials) were also mated to produce a low (noninhibi-
610 tor) latent inhibition line. Our previous studies have shown that worker progeny from in-
611 hibitor and non-inhibitor matings showed significant correlation in expression of latent in-
612 hibition to their parents (*12, 70*).

613 **Recombinant drones** Male honey bees (drones) were produced from a cross between
614 genetic lines selected for high and low expression of latent inhibition(*12, 17*). Hybrid
615 queens were reared from a cross of a queen from the inhibitor line instrumentally insemi-
616 nated(*71*) with sperm from a single drone from the noninhibitor line. These queens were
617 then allowed to mate naturally to increase longevity in a colony. Natural mating involves
618 mating with several different drones. However, since drones arise from unfertilized eggs,
619 the haploid (drone) genotype involves only recombination of the genotypes of the high
620 and low lines in the hybrid queen. A single hybrid queen was then selected to produce
621 drones. Sealed drone brood from the hybrid queen was placed in a small nucleus colony.
622 Queen excluder material (wire mesh that does not permit the passage of queens or
623 drones) was used to confine the emerging drones to the upper story. Upon emergence,
624 drones were individually marked on the thorax with enamel paint for later identification,
625 and then marked drones were co-fostered in a single outdoor colony until collected for
626 behavioral conditioning.

627 Mature drones were collected from the colony upon returning from mating flights during
628 the late afternoon the day before testing. Returning drones gathered on a piece of queen
629 excluder material blocking the colony entrance and were put into small wooden boxes
630 with queen excluder material on each side. They were then fed a small amount of honey
631 and placed in a queenless colony overnight. The following morning drones were secured
632 in a plastic harness using a small piece of duct tape (2 mm x 20 mm) placed between the
633 head and the thorax (*25*). All drones were then kept at room temperature for two hours.
634 They were then screened for their motivation to feed by lightly touching a small drop of

635 2.0 M sucrose solution to the antennae. Drones that extended their proboscis were se-
636 lected for training.

637 **Foragers** Female pollen foragers (workers) were captured at the colony entrance as de-
638 scribed above. Each bee was chilled to 4˚ C, restrained in a harness and fed to satiation
639 with 1.0 M sucrose. The next day bees were tested for motivation by stimulation of their
640 antennae with 2.0 M sucrose; bees that extended their proboscis were used in experi-
641 ments shown in Figures 3 and 4.

642 **Conditioning protocols**

643 *Familiarization*: Familiarization to the odor was done as described in (*6*). Restrained bees
644 were placed in individual stalls where a series of valves regulated odor delivery via a
645 programmable logic controller (PLC) (Automation Direct). Hexanol and 2-octanone were
646 used either as pure odorants or diluted to 2.0 M in hexanes with odor treatments counter-
647 balanced across animals. Odor cartridges were made by applying 3.0 µl of odorant onto
648 a piece of filter paper (2.5 x 35 mm) and inserting the filter paper into a 1 ml glass syringe.
649 The odor cartridge was then connected to a valve regulated by the PLC that shunted air
650 through the cartridge for 4 s once the automated sequence was initiated. Odor preexpo-
651 sure in all experiments involved 40 unreinforced presentations of odor for 4 s using a 5
652 min (Fig 1) or 30 s (Fig 3) intertrial interval (ITI). All odor cartridges were changed for fresh
653 ones after every 10 uses to avoid odor depletion(*72*). The use of pharmacological treat-
654 ment necessitated the use of a shorter ITI to avoid having the drug wear off before the
655 end of preexposure. Our previous studies have revealed that latent inhibition is robust
656 over this range of ITIs and odor concentrations (*6*).

657 *Proboscis Extension Response conditioning*: All PER learning paradigms used for testing
658 used a 5 min ITI. An acquisition trial consisted of a 4 s presentation of an odor, the condi-
659 tioned stimulus (CS, black or gray bars), followed by presentation of a 0.4 µl drop of 1.0
660 M sucrose solution, the unconditioned stimulus (US, triangles in Figs 1, 3 and 4). Three
661 seconds after onset of the CS the US was delivered using a Gilmont ® micrometer syringe.
662 The US was initially delivered by gently touching the antennae to elicit proboscis extension
663 and subsequent feeding. Once a bee began to extend its proboscis at the onset of CS
664 delivery, it was no longer necessary to touch the antennae prior to feeding.

665 We used two different procedures for testing latent inhibition after familiarization. For
666 evaluation of recombinant drones (Fig 1), subjects were conditioned to the familiarized
667 odor (X) as the CS over 6 forward pairing trials. The second procedure (Figs 3 and 4)
668 involved use of a within animal control protocol. After familiarization all subjects received
669 equivalent PER conditioning to 2 odors, one was the familiarized odor (X) and the other
670 was a novel odor (N) that honey bees can easily discriminate from the familiarized
671 odor(*73*). Odors were presented in a pseudorandomized order (NXXNXNNX or
672 XNNXNXXN) across trials such that equal numbers of animals received N or X on the first
673 trial. Pharmacological treatment required the use of a control procedure involving famil-
674 iarization to air to evaluate the degree to which expression of excitatory conditioning was
675 affected by drug treatment (Figs. 3A and 4A).
676 **Linkage analysis** Upon completion of the training paradigm, 523 drones were placed in
677 individual 1 ml micro-centrifuge tubes and stored at -70˚ C. Genomic DNA extraction fol-
678 lowed a standard protocol developed for honey bees (*74*). For SNP analysis, DNA was
679 selected from 94 drones that exhibited the highest level of latent inhibition (0, 1, 2, or 3
680 responses over the six test trials) and from another 94 drones that exhibited the lowest
681 level (5 or 6 responses). Analysis of the 188 samples was conducted by Sequenom, Inc.
682 San Diego, California.

683 The linkage map was built with a set of 311 SNP markers. The list of selected markers
684 was provided by Olav Rueppell from previous studies examining the genetic architecture
685 of foraging behavior and sucrose response thresholds (*75, 76*). The 74 SNPs segregating
686 in our mapping population were used for a QTL analysis. Map positions for markers in
687 linkage group one were determined using the *Apis mellifera* 4.0 genome. The software
688 MultiPoint 1.2 (http://www.mulitqtl.com) was used to determine the actual recombination
689 frequencies for markers in linkage group 1. Recombination frequencies were then con-
690 verted to centiMorgans using the Kosambi mapping function. The actual mapping dis-
691 tances in our mapping population were used in the QTL analysis. QTL analysis was per-
692 formed with MapQTL 4.0. Interval mapping and MQM mapping revealed one significant
693 QTL. Genomewide significant thresholds for $p<0.05$ (LOD = 2.6) and $p<0.01$ (LOD = 3.2)
694 were determined using an implemented permutation test (1000 runs).

695 **Pharmacological and DsiAmTyr1 treatments.** Yohimbine hydrochloride (Sigma) was
696 diluted to 10^{-4} M in saline (5 mM KCl, 10 mM NaH_2PO_4, pH 7.8). We chose a concentration
697 of yohimbine that has been shown to be effective in our previous study of its effect on
698 honey bee behavior (*42*). One µl of drug or saline alone was injected into the brain through
699 the median ocellus using a Hamilton syringe (Hamilton; Reno, NV). Training began 15
700 min after injection, as this time has been shown to be effective in other drug studies using
701 the same methodology (*6, 48, 77*).

702 For DsiRNA studies, we used sequences and protocols developed previously for a
703 study of *Amtyr1* receptor distribution in the brain, which in that study were used to show
704 that the anti-*Amtyr1* antibodies specifically recognized the receptor(*28*). We used a Dicer-
705 substrate small interfering (Dsi) RNA of the *AmTyr1* receptor (NCBI Reference Sequence:
706 NM_001011594.1) to knockdown *AmTyr1* mRNA receptor in the brain. We used the mix-
707 ture of three DsiAmTyr1 constructs designed by the tool in IDT technology(*28*) (Table 1).
708 As a control we used a scrambled (dsiScr) version of the *Amtyr1* sequence. A 138 nano-
709 liter injection of a 100microM mixture of dsiAmTyr1 or dsiScr (Nanoinject 2000) was made
710 into the middle ocellus 18-20 hours before behavioral tests. All injections were done blind
711 so that the investigator doing behavioral tests was not aware of the content of the injection.
712 After the tests brains without optic lobes were dissected out and homogenized each in
713 TRIzol (Invitrogen) (N=27 for bees injected with dsiScr and N=32 for bees injected with
714 DsiAmTyr1). Then, the total mRNA from each injected brain was extracted separately
715 using the manufacturer's protocol for TRIzol method (Invitrogen). Contaminating genomic
716 DNA was removed using DNA-free™ kit (Ambion, AM1906). RNA quantity and purity was
717 evaluated using a NanoDrop (NanoDrop 2000). Expression of AmTyr1 was quantified us-
718 ing QuantiFAST SYBR Green RT-PCR kit (QIAGEN) on Applied Biosystem 7900 cycler
719 (ASU DNA Facilities) with the protocol provided by the kit for a 384-well plate. The primers
720 for quantitative real-time PCR assays were: AmTyr1_F 5'-
721 GTTCGTCGTATGCTGGTTGC-3', AmTyr1_R 5'- GTAGATGAGCGGGTTGAGGG-3' and
722 for reference gene AmActin_F 5'- TGCCAACACTGTCCTTTCTG-3' AmActin_R 5'-
723 AGAATTGACCCACCAATCCA-3'(*78*).

Table 1

Nucleotide sequences of sense and antisense strands of control DsiSCR and AmTyr1 DsiRNA.

DsiRNA	Sequences
DsiScr	5′- GAGUCCUAAGUUAACCAAGUCACAGCA-3 3′- CUCAGGAUUCAAUUGGUUCAGUGUCGU-5′
DsiTyr1_N	5′-AGCGUGACGUUGGAUUGACGAGAGC-3′ 3′-CCUCGCACUGCAACCUAACUGCUCUCG-5′
DsiTyr1_T1	5′-CCUGUGCAAAUUGUGGCUAACCUGC-3′ 3′-GUGGACACGUUUAACACCGAUUGGACG-5′
DsiTyr_C	5′-CAACGCUUGUUUAUUGCAUCUAUCG-3′ 3′-CCGUUGCGAACAAAUAACGUAGAUAGC-5′

All injections were done blind so that the investigator doing behavioral tests was not aware of the content of the injection.

Electrophysiological recordings from the antennal lobe. Extracellular recordings were performed in the antennal lobes with a 16-channel probe (NeuroNexus, Ann Arbor, MI). Spike waveforms were digitized with a RZ2 system at a sampling rate of 20KHz (Tucker-Davis Technologies, Alachua, FL). After a stable recording was achieved, the honeybee preparation was first stimulated with two presentations of each of the following odors: 1-hexanol (Hex) and 2-octanone (Oct). The duration of each pulse was 4 sec, and two minutes of recovering time were allowed between two pulses. During the pre-exposure phase, 40 pulses of Oct were delivered with inter-pulse interval of 60 sec, after which 10 min recovery was given before testing. Upon completion of each experiment, extracellular spike waveforms are exported to Offline Sorter program (Plexon Inc, Dallas, TX) which classifies the similar waveforms into individual clusters (units) based on the relatedness of waveforms' projection onto a 3D space derived from the first three principle components that capture the most variation of the original waveforms. To increase the discriminating power, the original waveforms are grouped in a tetrode configuration, matching the physical design of the recording probe, i.e. 16 recording sites are distributed in two shanks in a block design of 2x4. Each block is called a tetrode. Statistical separation of waveform

743 clusters, representing individual neurons or units, is aided with visual inspection, all im-
744 plemented in the Offline Sorter program. Once satisfied with the clustering results, the
745 time stamps of waveforms are then exported to Neuroexplorer program (Plexon Inc, Dal-
746 las, TX) and Matlab (Mathworks, Natick, MA) for further analysis.

747 Yohimbine (Millipore-Sigma, St. Louis, MO.), was diluted in saline (50 μM), which was
748 perfused into the head capsule through a T-tube switch. Repeated stimulation with Oct
749 started fifteen minutes after perfusion; by then the slowing-down of spiking activities were
750 often noticeable. Care was taken not to introduce any air bubble into the tubing when
751 switching from the syringe containing saline to the syringe containing the yohimbine solu-
752 tion. The water level in the two syringes was intentionally kept the same in order to main-
753 tain a similar perfusing rate upon switching. The drug solution was kept flowing through
754 the honeybee preparation until the end of protocol, which usually lasted for about 2 hours.
755 No saline wash was attempted in this protocol due to the long time required for the re-
756 cording sessions.

757 **Sequencing the *Amtyr1* region of the genome**. We studied single nucleotide polymor-
758 phisms (SNPs) in full-genome sequences of eight *A. mellifera* workers (four high pollen
759 hording and four low pollen hording). For each individual, Illumina short reads were
760 mapped against the *Apis mellifera* genome assembly version 4.5 (*79*) using bwa version
761 0.5.9-r16 (*80*). An average 25x genome coverage per individual allowed the identification
762 of high-quality SNPs in each individual against the reference genome. SNPs were identi-
763 fied with SAMtools version 0.1.17-r973:277 (*81*) enforcing a minimum quality score of 20
764 (base call accuracy ≥ 99%).

765 **Statistical analysis.** To analyze the effects in behavioral experiments, we used a
766 generalized linear model with binomial error distribution and logit transformation to
767 perform a logistic regression. The response variable is binomal (0,1). Trial is an ordered
768 variable. We were most interested in testing the hypothesis that injection of yohimbine
769 and dsiRNA before familiarization treatment would impact latent inhibition, so we focused
770 on the interactions between trial, injection, injection time (before pre-exposure or before
771 acquisition), and odor (novel or pre-exposed odor). To explore significant interactions

further, we performed a tukey post hoc test using the package emmeans. All analyses were performed in R version 4.2.0 using RStudio version 2022.07.1.

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