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 increases 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 regulates inhibition, such that without a functional AmTYR1 protein inhibition becomes strong and suppresses sensory responses that drive Hebbian plasticity underlying latent inhibition. 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.

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

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 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

74 identified in independent mapping studies as being important for latent inhibition(*19*) as
75 well as for sugar and pollen preferences in foragers (*20, 21*). Disruption of a tyramine
76 receptor encoded by *Amtyr1* in this region *increases* latent inhibition, suggesting that in-
77 tact signaling via the *Amtyr1* pathway is important for modulating inhibition. Furthermore,
78 electrophysiological analyses combined with blockade of the AmTYR1 receptor in the an-
79 tennal lobe – the first synaptic center along the olfactory pathway – decreased antennal
80 lobe responsiveness to odor. Finally, sequencing the gene failed to reveal mutations in
81 the coding regions that would affect protein function, leading to the conclusion that varia-
82 tion across workers could arise from differential gene expression through transcriptional
83 regulation. We discuss how these data strongly imply a functional role for *Amtyr1* signaling
84 in regulating expression of attention via latent inhibition. These findings are also important
85 for understanding the strategies colonies use to explore for and exploit pollen and nectar
86 resources(*22*).

87 **Results**

88 We used two genetic lines of honey bees that had been bred for high (inhibitor) or low
89 (noninhibitor) expression of latent inhibition. These lines were independently selected us-
90 ing identical methods to a previous study that had successfully bred high and low
91 lines(*19*). We evaluated 523 recombinant drones generated from a single hybrid queen
92 produced from a cross between a drone from a noninhibitor line and a queen from an
93 inhibitor line (Fig. 1). Honey bee drones are ideal for behavior genetic studies because
94 they are haploid progeny that develop from an unfertilized egg laid by the queen. We then
95 selected 94 high and 94 low performing drones for the Quantitative Trait Locus (QTL)
96 analysis, which identified one significant locus (Fig. 2A). The QTL mapped to the same
97 genomic region identified in a previous study of latent inhibition (called '*lrn1'*) using an
98 independent inhibitor and noninhibitor cross and different (RAPD-based) genetic markers
99 (*19*). This is the same genomic region that has been identified in studies of foraging pref-
100 erences of honey bees (*21, 23*), where it has been called *pln2* for its effect on pollen
101 versus nectar preferences and in modulating sensitivity to sucrose (*24*). Clearly this ge-
102 nomic region has major effects on several foraging-related behaviors.

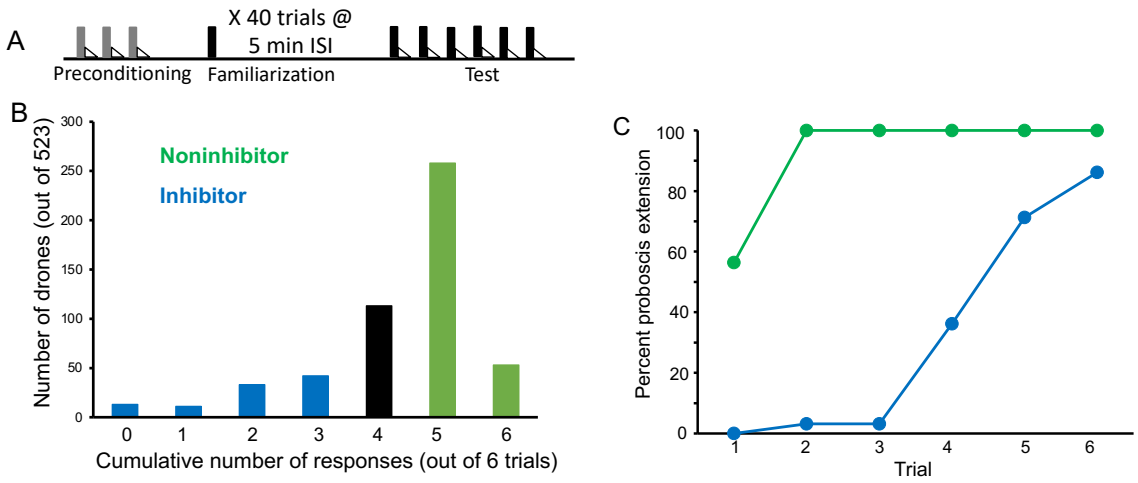
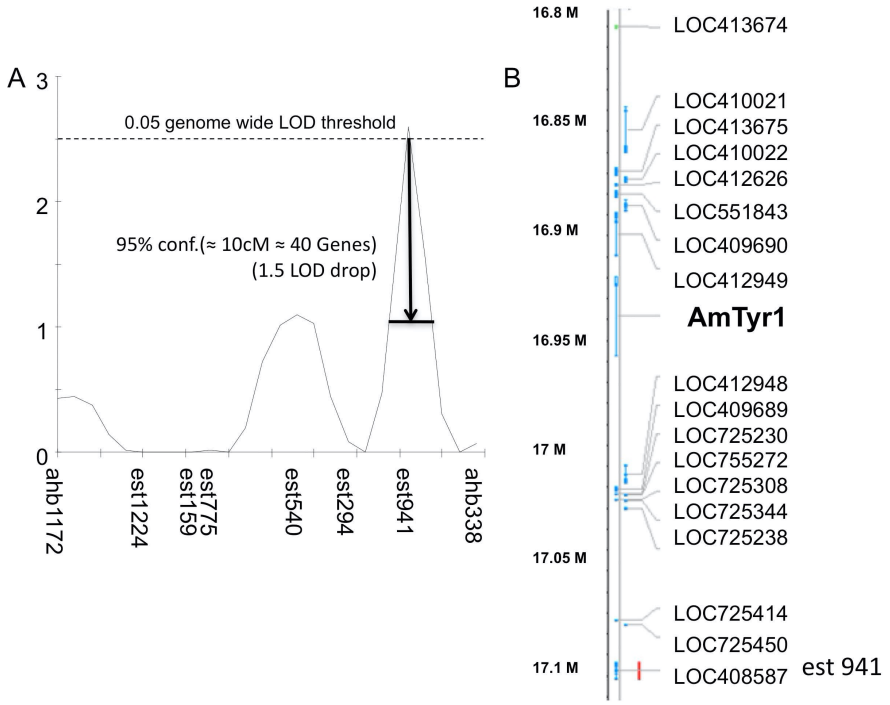


Fig. 1. Evaluation and selection of drones from an F1 queen. **(A)** Drone honey bees were first evaluated by conditioning them over three trials to an odor (A; gray bars) followed by sucrose reinforcement (triangles) in a way that produces robust associative conditioning expressed as odor-induced proboscis 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 preexposure 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*).



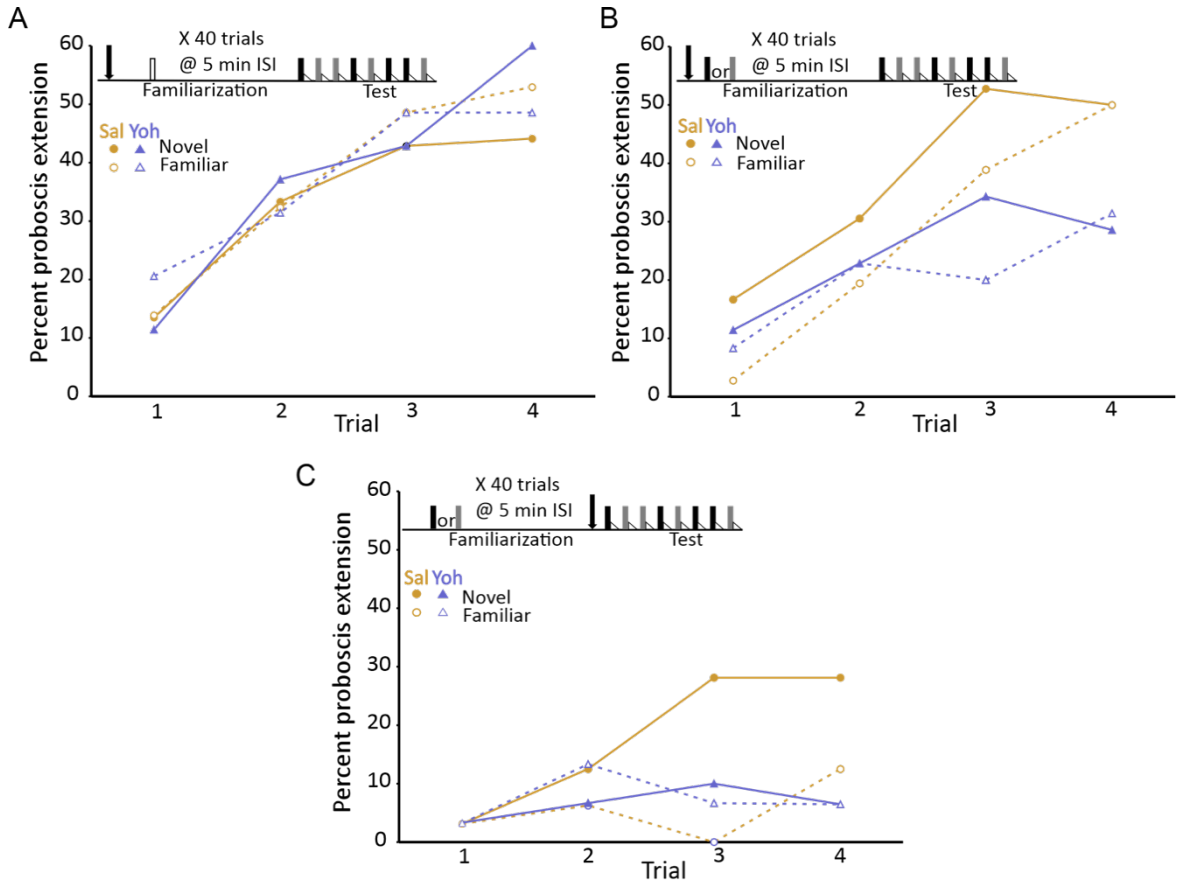
143 **Fig. 2. Single-Nucleotide Polymorphism (SNP) mapping of high and low recombinant**
144 **drones. (A)** Markers from linkage group 1.55 surrounding one significant QTL (est941 with a LOD
145 score of 2.6). **(B)** Partial list of genes within 10 cM of the marker showing the location of *Amtyr1*.

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

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

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

174 appetitive conditioning (*25*). Latent inhibition would be evident if responses to odor X were
175 lower than the responses to the novel odor N. Injections of yohimbine directly into brains
176 occurred either prior to the familiarization phase (Fig. 3A,B) or prior to the test phase (Fig.
177 3C).



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

df=3, p<0.001). None of the remaining effects (odor, injection or any of the interaction terms) were significant (p>0.05). **(B)** As in **A**, except both groups (orange saline: n=36; blue yohimbine: n=36) in this experiment were familiarized to odor; each odor (gray and black boxes; see Methods) was familiarized in approximately half of the animals in each injection group. In this design, each individual was equivalently conditioned to both odors during the test phase; latent inhibition is evident when the response to the novel odor is greater than to the familiar odor. Injection was prior to odor familiarization. **(C)** As in **B**, except injection of saline (n=32) or yohimbine (n=30) occurred prior to the test phase. Statistical analysis of data sets in **B** and **C** yielded a significant interaction (X^2=7.4, df=1, p <0.01) between injection (saline vs yohimbine) and odor (novel vs familiar) that was the same in both experiments, as judged by the lack of a significant odor*injection*experiment interaction term (p>0.05). There was a higher response to the novel odor than to the familiar odor, but only in the saline injected groups. The lower rate of acquisition in **C** (X^2=64.0,1,p<0.01) could be due to performance of this experiment at a different time of year, or to injections immediately prior to testing, which affects levels PER conditioning in honey bees but leaves intact relative differences between groups (*45*).

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

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

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

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

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

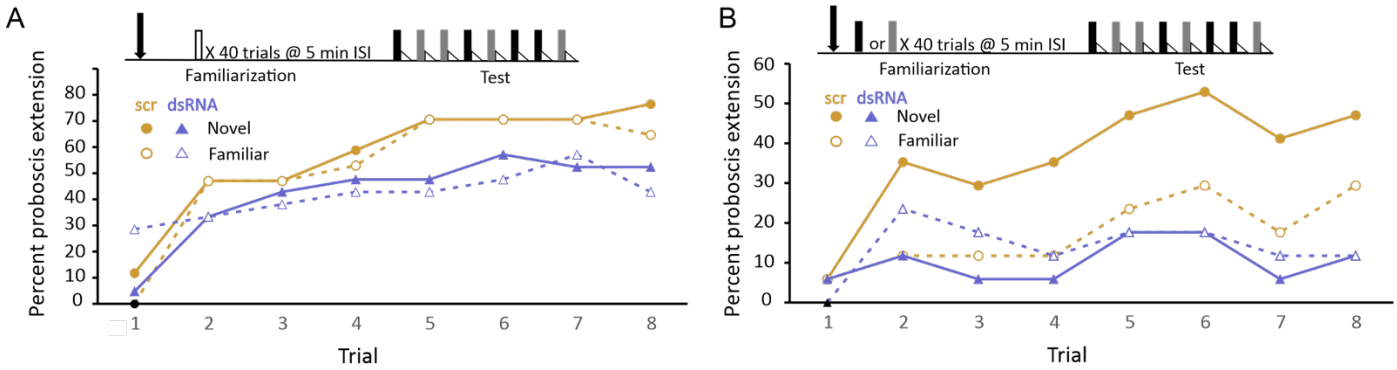


Fig. 4 Disruption of translation of the tyramine receptor by DsiRNA increased expression of latent inhibition. This experiment was identical to that shown in Fig 3A,B, except injections were performed with a mixture of Dsi *Amtyr1*RNA (dsRNA; arrow) 24 hour prior to behavioral training and testing. The 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, because blockade and disruption of *Amtyr1*

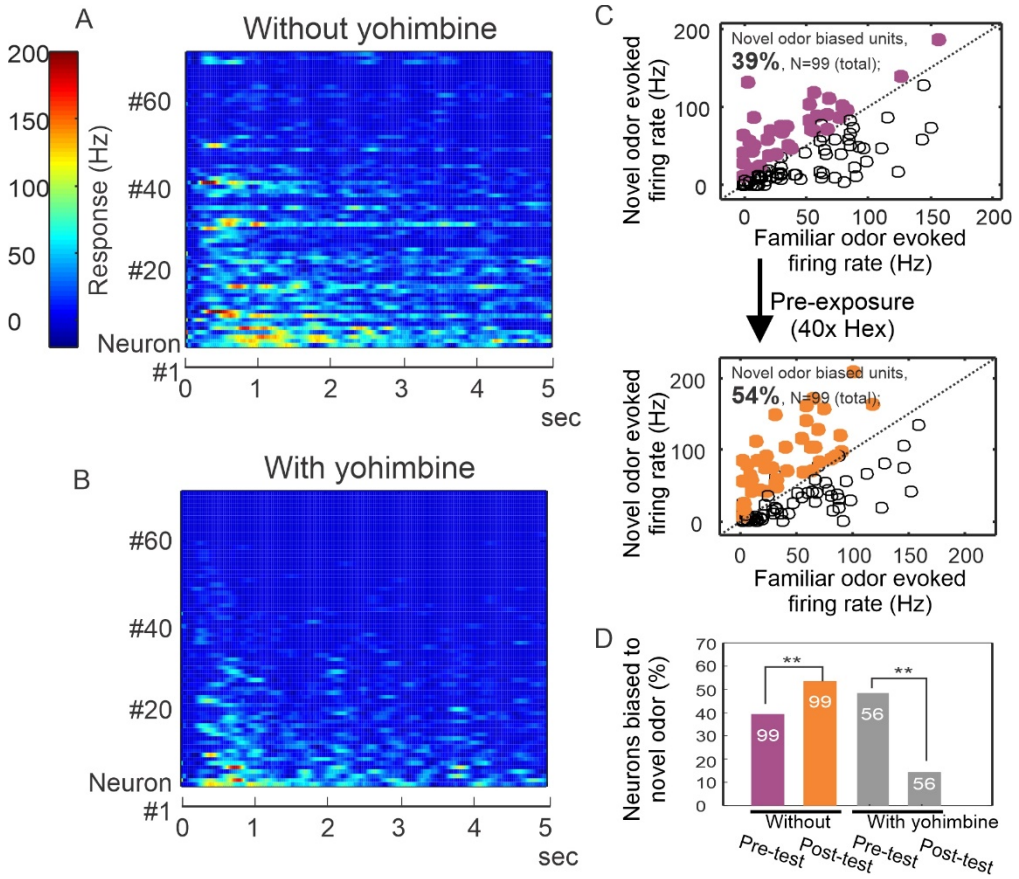
did not attenuate latent inhibition. Instead, treatment with yohimbine or *Amtyr1* DsiRNA *enhanced* latent inhibition. This result is consistent with *Amtyr1* modulating inhibition involved in, for example, identified inhibitory processes in the antennal lobes(*50*). Specifically, and as we propose below in a model, it would prevent the inhibition from becoming too strong, and possibly keep it at a set point between very strong and very weak.

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

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

We therefore chose to analyze the effect of yohimbine treatment on odor processing in the antennal lobes by recording electrophysiological responses to odors prior to and after yohimbine treatment. This first experiment did not employ familiarization to odor. We used yohimbine in these experiments because of the more rapid onset (minutes versus hours) compared to DsiRNA treatment. Prior to yohimbine treatment, recordings from 71 units across 4 animals revealed responses to odors that ranged from no detectable change in spike activity with odor presentation to a robust increase in spiking activity (Fig.5A). After yohimbine treatment, responses decreased precipitously, although some spiking activity was still detectable (Fig. 5B). This decrease in response is consistent with AmTYR1 being involved in regulation of inhibition in networks of the antennal lobe, which are the first order processing centers for odors. Specifically, intact/functioning AmTYR1 receptors on sensory axon terminals(*28*) could potentially decrease excitatory drive onto inhibitory networks and possibly keep inhibition modulated between two extremes. Hypothetically at

306 least, when AmTYR1 is blocked by yohimbine, excitation of inhibitory networks in the an-
307 tennal lobe increases and drives down PN responses.



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

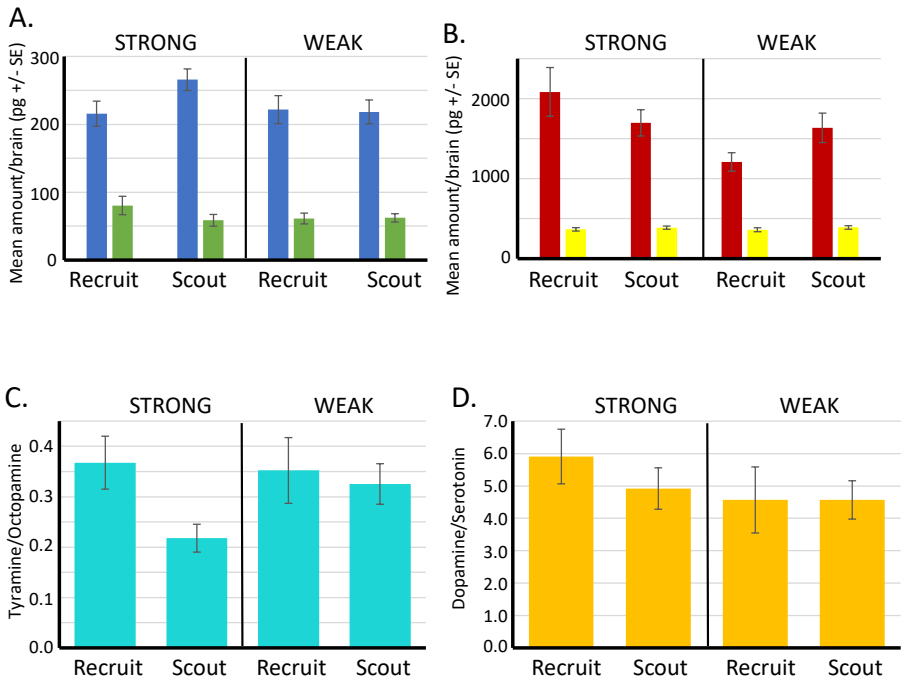
with the familiar odor (*McNemar* test with Yates's correction, d.f.=1, Chi-square=11.13, $p<0.001$) (asterisks on grey bars, N=56).

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

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

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

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



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

dopamine/serotonin did not differ in either the strong or weak groups (p>0.05). Sample sizes the same as in C.

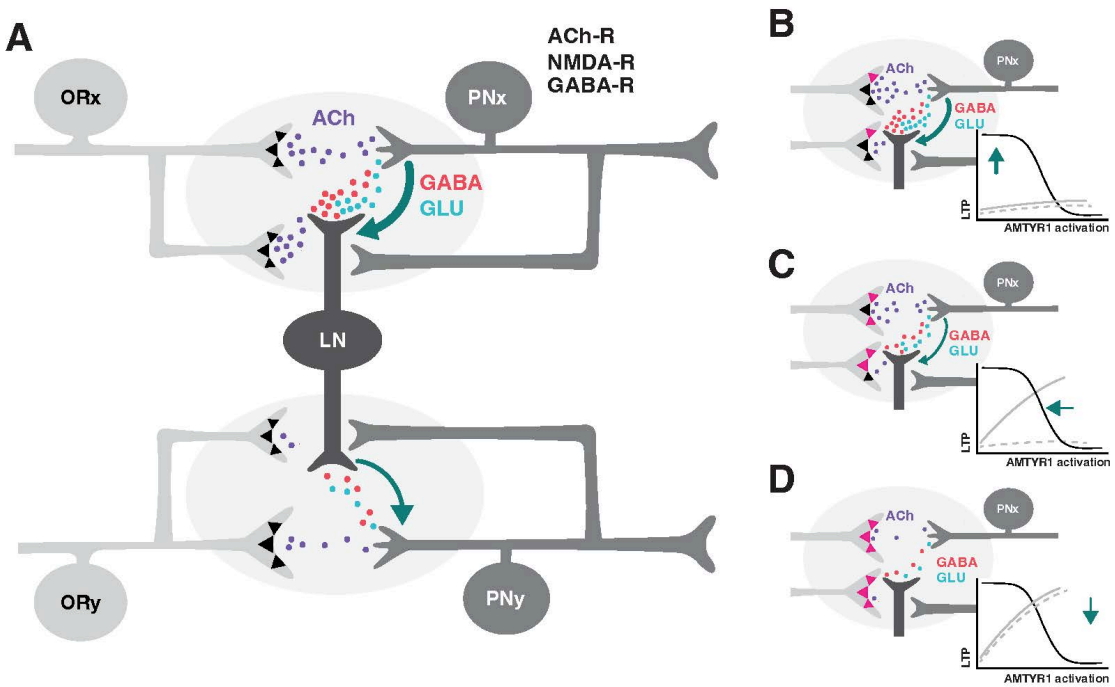
Discussion

Our results have identified the neural underpinnings of an important form of learning and memory in the brain. All animals need to learn about stimuli in their environment. Latent inhibition is important for redirecting limited attention capacity away from unimportant, inconsequential stimuli and refocusing it toward novel stimuli about which the animal knows little or nothing. Two independent QTL mapping studies have now identified the genetic locus that contains *Amtyr1* as important for regulating individual variation in attention(*19*). There are other loci in the genome that show associations with the behavior, and there are also other unidentified genes in the same locus. Nevertheless, our manipulation of *Amtyr1* function using both pharmacology and DsiRNA treatments confirm its association with behavioral expression of latent inhibition.

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

Second, the effects of *Amtyr1* specifically on expression of latent inhibition likely arise by combining its expression in sensory as well as more central areas of the brain. That is, it is unlikely that there is a single locus in the brain that underlies latent inhibition. We have shown for honey bees(*28*), for example, that AmTYR1 is expressed on presynaptic terminals of Olfactory Sensory Neurons in the antennal lobes, where they provide excitation to dendrites of Local GABAergic Inhibitory Interneurons (LN) and Projection Neurons (PN) (Fig. 7A). AmTYR1 is also expressed on presynaptic terminals of Projection Neuron axons that terminate in the mushroom body calyces as well as on some intrinsic neurons of the

403 calyces. In the antennal lobe, where our electrophysiological and imaging studies have
404 focused (*51, 52, 56, 57*), familiarization to an odor causes the neural representation of a
405 mixture that contains the familiar odor to become more like novel odors in the mixture
406 (*52*). We assume, but have yet to show experimentally, that this bias combines with how
407 AmTYR1 affects processing in the mushroom bodies, where olfactory information con-
408 verges 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*).



412

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

420 glutamate (green) onto synapses with PNs. NMDA receptors on PNs potentiate the LN/PN
421 synapses via a retrograde signal (green arrows) to LNs. This hebbian plasticity increases
422 inhibition of PNs by LNs and thus decreases excitation of the PN. LN/PN coactivation in
423 other glomeruli is either non-existent (e.g.(*58*)), too low to produce potentiation of the
424 LN/PN synapse, or strong enough to produce inhibition to odors other than the familiar
425 odor. AmTYR1 receptors on presynaptic terminals of ORNs (*28*) are shown as black tri-
426 angles. Here we do not show the source for tyramine. We assume it could be from the
427 VUM neuron, which has terminals in the cortex of every glomerulus, where ORN terminals
428 overlap with PN and LN dendrites(*59*). B-D. Increasing activation of AmTYR1 (pink trian-
429 gles) progressively decreases release of ACh and lowers coactivation of LNs and PNs.
430 Decreased release of glutamate reaches a threshold (D) below which it fails to modify the
431 LN/PN synapse via a decreased retrograde signal, although ACh release might still acti-
432 vate the glomeruli. Graphs inset in each figure show activation of AmTYR1 (x-axis) verses
433 LTP-based hebbian plasticity (y-axis). Low activation of AmTYR1 produces high coacti-
434 vation-based LTP (B) and vice-versa (D). Arrows show the hypothetical relationship in
435 each figure. Gray lines represent the predicted acquisition levels to novel (solid) and fa-
436 miliar (dashed) odors given the strength of Hebbian plasticity in each figure. For these
437 lines the x-axis would be 'Trial' and y-axis 'Percent proboscis extension', as in Figs. 3 and
438 4.

439

440 The precise relationship of *Amtyr1* to latent inhibition is different from what is normally
441 expected from disruption of a gene that underlies a behavior. We expected that disruption
442 of *Amtyr1* function would reduce or eliminate latent inhibition; that is, learning about a
443 familiar odor (X) would rise to equal learning about the novel odor. Instead, latent inhibition
444 was strengthened so much so that it appears to generalize even to the novel odor. This
445 reduction cannot be explained by nonspecific – e.g. toxic – effects of treatment, because
446 the same treatments did not reduce excitatory conditioning in the absence of familiariza-
447 tion to an odor. Moreover, the same effect was evident using two very different means
448 for disruption of *Amtyr1* signaling.

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

A model of how *amtyr1* modulates inhibition in antennal lobe networks

We can now propose a model for how *Amtyr1* acts in the antennal lobes, and possibly also as an important component for encoding latent inhibition in distributed networks in the brain. The model would have to explain why air (mechanosensory) stimulation alone does not reduce subsequent acquisition to odors (Figs. 3A, 4A) as much as familiarization to an odor (Figs. 3B,C 4B). Mechanosensory stimulation produces fast, transient responses in antennal lobe glomeruli, whereas odor stimulation produces more robust, longer lasting responses(*60, 61*). These differences in response could underlie the difference in response to air versus odor in our analyses.

We propose that the strong reduction specific to odor preexposure occurs by amplifying hebbian plasticity between PNs and LNs in the antennal lobe networks (Fig. 7A). A computational model of the honey bee antennal lobe identified hebbian plasticity at Local GABAergic Inhibitory Interneuron (LN) synapses onto Projection Neurons (PN) as the most likely locus of plasticity to give rise to the observed biasing of an odor mixture to be less like a familiar odor (*52*). Work with fruit flies also showed that glutamate/NMDA receptor dependent hebbian plasticity at the same synapses underlies odor habituation ((*58*); the same process as latent inhibition just differently named).

In our model (Fig. 7A-D), coactivation of PNs and LNs would produce plasticity at the LN/PN synapses. At moderate levels (Fig. 7C) this plasticity it would give rise to normal latent inhibition in our high lines just as it does in fruit flies. Accordingly, lack of activation of AmTYR1 (Fig. 7B) would lead to strong input from sensory axon terminals that would maximally activate LNs and PNs, thus leading to strong Hebbian plasticity at LN-to-PN synapses. That is, it would lead to the strong latent inhibition in our manipulations that

479 blocked or disrupted *Amtyr1* signaling. Strong activation of AmTYR1 would weaken the
480 activity and reduce or prevent plasticity (Fig. 7D), and hence suppress expression of latent
481 inhibition (such as in our genetic lines that do not show strong latent inhibition; (*12*)). How
482 well this model works might depend on a nonlinear thresholding function for coactivation
483 that drives hebbian plasticity (*Amtyr1*-LTP relationships and expected learning curves
484 shown in Figs. 7B-D).

485 Furthermore, strong activation of lateral projections of the inhibitory interneuron (Fig.
486 7A) might cause generalization of latent inhibition to novel odors, as we have observed
487 with disruption of *Amtyr1* signaling. Many of the odorants that have been used to study
488 olfaction and olfactory learning in honey bees have neural activity patterns that partially
489 overlap (*62*), such as with the representations for hexanol and 2-octanone. For each odor,
490 PNs in a few of the 160+ glomeruli of the antennal lobe are highly activated, and a subset
491 of other PNs are activated to a lesser degree. Hypothetically at least, if the familiar odor
492 activates some of the PNs from glomeruli that also code for the novel odor, then coacti-
493 vation of those PNs with lateral inhibition (as well as with local inhibition) could cause
494 Hebbian plasticity at those synapses too. Under normal circumstances the lower activa-
495 tion of those PNs might be too low to produce latent inhibition (Fig. 7D). But when *Amtyr1*
496 signaling is too low or disrupted (Fig 7 B), coactivation would increase enough to drive the
497 plasticity.

498 Finally, it remains to be determined what the source for tyramine could be, and which
499 of the local interneuron types might be the ones mediating latent inhibition in the antennal
500 lobes. VUM neurons are an obvious possibility for the source of tyramine, since tyramine
501 is a direct precursor to octopamine released by VUM neurons(*63*). However, this hypoth-
502 esis would depend on tyramine being released at almost constant, low levels without stim-
503 ulation of VUM by taste receptors sensitive to sugars. The likely candidates for local in-
504 terneurons might be the heteroLNs(*64*), which receive excitatory inputs in one glomerulus
505 and transmit inhibition to other glomeruli. Computational modeling suggests that the inter-
506 glomerular connectivity of heteroLNs should be based on 'functional networks' defined by
507 overlapping glomerular activity patterns to similar odors(*50*).

508 **Ideas and Speculation**

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

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

Interestingly, we have identified a potential interaction in the ratio of tyramine to octopamine between foraging role (scouts versus recruits) and expression of latent inhibition. The lower tyramine-to-octopamine ratios in scouts would potentially activate this receptor even less that it would normally be, yielding stronger inhibition according to the model described above. Further analyses need to test this prediction in more detail and evaluate 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, 65*). 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(*65*). We have therefore proposed that genetic variation for gene regulation, 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 reinforcement in a way that normally produces robust associative conditioning (*25*). Strong

latent inhibition should slow the rate of learning to X. Drones and queens that exhibited this 'inhibitor' phenotype (defined as zero or one response to X over six conditioning trials) (*12*) were mated using standard instrumental insemination techniques (*66*) for honey bees to create a high (inhibitor) latent inhibition line. Drones and queens that learned X quickly (five positive responses to X over six trials) were also mated to produce a low (noninhibitor) latent inhibition line. Our previous studies have shown that worker progeny from inhibitor and non-inhibitor matings showed significant correlation in expression of latent inhibition to their parents (*12, 67*).

Recombinant drones Male honey bees (drones) were produced from a cross between genetic lines selected for high and low expression of latent inhibition(*12, 17*). Hybrid queens were reared from a cross of a queen from the inhibitor line instrumentally inseminated(*68*) with sperm from a single drone from the noninhibitor line. These queens were then allowed to mate naturally to increase longevity in a colony. Natural mating involves mating with several different drones. However, since drones arise from unfertilized eggs, the haploid (drone) genotype involves only recombination of the genotypes of the high and low lines in the hybrid queen. A single hybrid queen was then selected to produce drones. Sealed drone brood from the hybrid queen was placed in a small nucleus colony. Queen excluder material (wire mesh that does not permit the passage of queens or drones) was used to confine the emerging drones to the upper story. Upon emergence, drones were individually marked on the thorax with enamel paint for later identification, and then marked drones were co-fostered in a single outdoor colony until collected for behavioral conditioning.

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

596 2.0 M sucrose solution to the antennae. Drones that extended their proboscis were se-
597 lected for training.

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

603 **Conditioning protocols**

604 *Familiarization*: Familiarization to the odor was done as described in (*6*). Restrained bees
605 were placed in individual stalls where a series of valves regulated odor delivery via a
606 programmable logic controller (PLC) (Automation Direct). Hexanol and 2-octanone were
607 used either as pure odorants or diluted to 2.0 M in hexanes with odor treatments counter-
608 balanced across animals. Odor cartridges were made by applying 3.0 µl of odorant onto
609 a piece of filter paper (2.5 x 35 mm) and inserting the filter paper into a 1 ml glass syringe.
610 The odor cartridge was then connected to a valve regulated by the PLC that shunted air
611 through the cartridge for 4 s once the automated sequence was initiated. Odor preexpo-
612 sure in all experiments involved 40 unreinforced presentations of odor for 4 s using a 5
613 min (Fig 1) or 30 s (Fig 3) intertrial interval (ITI). All odor cartridges were changed for fresh
614 ones after every 10 uses to avoid odor depletion(*69*). The use of pharmacological treat-
615 ment necessitated the use of a shorter ITI to avoid having the drug wear off before the
616 end of preexposure. Our previous studies have revealed that latent inhibition is robust
617 over this range of ITIs and odor concentrations (*6*).

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

We used two different procedures for testing latent inhibition after familiarization. For evaluation of recombinant drones (Fig 1), subjects were conditioned to the familiarized odor (X) as the CS over 6 forward pairing trials. The second procedure (Figs 3 and 4) involved use of a within animal control protocol. After familiarization all subjects received equivalent PER conditioning to 2 odors, one was the familiarized odor (X) and the other was a novel odor (N) that honey bees can easily discriminate from the familiarized odor(*70*). Odors were presented in a pseudorandomized order (NXXNXNNX or XNNXNXXN) across trials such that equal numbers of animals received N or X on the first trial. Pharmacological treatment required the use of a control procedure involving familiarization to air to evaluate the degree to which expression of excitatory conditioning was affected by drug treatment (Figs. 3A and 4A).

Linkage analysis Upon completion of the training paradigm, 523 drones were placed in individual 1 ml micro-centrifuge tubes and stored at -70˚ C. Genomic DNA extraction followed a standard protocol developed for honey bees (*71*). For SNP analysis, DNA was selected from 94 drones that exhibited the highest level of latent inhibition (0, 1, 2, or 3 responses over the six test trials) and from another 94 drones that exhibited the lowest level (5 or 6 responses). Analysis of the 188 samples was conducted by Sequenom, Inc. San Diego, California.

The linkage map was built with a set of 311 SNP markers. The list of selected markers was provided by Olav Rueppell from previous studies examining the genetic architecture of foraging behavior and sucrose response thresholds (*72, 73*). The 74 SNPs segregating in our mapping population were used for a QTL analysis. Map positions for markers in linkage group one were determined using the *Apis mellifera* 4.0 genome. The software MultiPoint 1.2 (http://www.mulitqtl.com) was used to determine the actual recombination frequencies for markers in linkage group 1. Recombination frequencies were then converted to centiMorgans using the Kosambi mapping function. The actual mapping distances in our mapping population were used in the QTL analysis. QTL analysis was performed with MapQTL 4.0. Interval mapping and MQM mapping revealed one significant QTL. Genomewide significant thresholds for $p<0.05$ (LOD = 2.6) and $p<0.01$ (LOD = 3.2) were determined using an implemented permutation test (1000 runs).

656 **Pharmacological and DsiAmTyr1 treatments.** Yohimbine hydrochloride (Sigma) was
657 diluted to 10^{-4} M in saline (5 mM KCl, 10 mM NaH_2PO_4, pH 7.8). We chose a concentration
658 of yohimbine that has been shown to be effective in our previous study of its effect on
659 honey bee behavior (*42*). One µl of drug or saline alone was injected into the brain through
660 the median ocellus using a Hamilton syringe (Hamilton; Reno, NV). Training began 15
661 min after injection, as this time has been shown to be effective in other drug studies using
662 the same methodology (*6, 48, 74*).

663 For DsiRNA studies, we used sequences and protocols developed previously for a
664 study of *Amtyr1* receptor distribution in the brain, which in that study were used to show
665 that the anti-*Amtyr1* antibodies specifically recognized the receptor(*28*). We used a Dicer-
666 substrate small interfering (Dsi) RNA of the *AmTyr1* receptor (NCBI Reference Sequence:
667 NM_001011594.1) to knockdown *AmTyr1* mRNA receptor in the brain. We used the mix-
668 ture of three DsiAmTyr1 constructs designed by the tool in IDT technology(*28*) (Table 1).
669 As a control we used a scrambled (dsiScr) version of the *Amtyr1* sequence. A 138 nano-
670 liter injection of a 100microM mixture of dsiAmTyr1 or dsiScr (Nanoinject 2000) was made
671 into the middle ocellus 18-20 hours before behavioral tests. All injections were done blind
672 so that the investigator doing behavioral tests was not aware of the content of the injection.
673 After the tests brains without optic lobes were dissected out and homogenized each in
674 TRIzol (Invitrogen) (N=27 for bees injected with dsiScr and N=32 for bees injected with
675 DsiAmTyr1). Then, the total mRNA from each injected brain was extracted separately
676 using the manufacturer's protocol for TRIzol method (Invitrogen). Contaminating genomic
677 DNA was removed using DNA-free™ kit (Ambion, AM1906). RNA quantity and purity was
678 evaluated using a NanoDrop (NanoDrop 2000). Expression of AmTyr1 was quantified us-
679 ing QuantiFAST SYBR Green RT-PCR kit (QIAGEN) on Applied Biosystem 7900 cycler
680 (ASU DNA Facilities) with the protocol provided by the kit for a 384-well plate. The primers
681 for quantitative real-time PCR assays were: AmTyr1_F 5'-
682 GTTCGTCGTATGCTGGTTGC-3', AmTyr1_R 5'- GTAGATGAGCGGGTTGAGGG-3' and
683 for reference gene AmActin_F 5'- TGCCAACACTGTCCTTTCTG-3' AmActin_R 5'-
684 AGAATTGACCCACCAATCCA-3'(*75*).

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

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

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

718 **Sequencing the *Amtyr1* region of the genome**. We studied single nucleotide polymor-
719 phisms (SNPs) in full-genome sequences of eight *A. mellifera* workers (four high pollen
720 hording and four low pollen hording). For each individual, Illumina short reads were
721 mapped against the *Apis mellifera* genome assembly version 4.5 (*76*) using bwa version
722 0.5.9-r16 (*77*). An average 25x genome coverage per individual allowed the identification
723 of high-quality SNPs in each individual against the reference genome. SNPs were identi-
724 fied with SAMtools version 0.1.17-r973:277 (*78*) enforcing a minimum quality score of 20
725 (base call accuracy ≥ 99%).

726 **Statistical analysis.** To analyze the effects in behavioral experiments, we used a
727 generalized linear model with binomial error distribution and logit transformation to
728 perform a logistic regression. The response variable is binomal (0,1). Trial is an ordered
729 variable. We were most interested in testing the hypothesis that injection of yohimbine
730 and dsiRNA before familiarization treatment would impact latent inhibition, so we focused
731 on the interactions between trial, injection, injection time (before pre-exposure or before
732 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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Funding

National Institutes of Health NIGMS (R01GM113967)

- Brian H Smith

National Science Foundation CRCNS (2113179)

- Brian H Smith

National Science Foundation NeuroNex (1559632)

- Brian H Smith

Department of Energy (SC0021922)

- Brian H Smith

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.