Title: Tyramine and its AmTYR1 receptor modulate attention in honey bees (*Apis mellifera*)

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One-sentence summary: A biogenic amine receptor underlies attention that is the basis for exploration/exploitation tradeoffs during foraging

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*. We then show that disruption of *Amtyr1* signaling either pharmacologically or genetically increases expression of latent inhibition without affecting appetitive conditioning. 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 in general. 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 reinforcement pathway for this type of non-associative learning, which we have shown also underlies potentially adaptive intracolonial learning differences among individuals that in combination benefit colony survival.

Main text: 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 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 *increases* latent inhibition, suggesting that intact signaling via the *Amtyr1* pathway is important for modulating inhibition. 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. 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 regulating 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*).

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 (*23, 24*), where it has been called *pln2* for its effect on pollen versus nectar preferences and in modulating sensitivity to sucrose (*25*). Clearly this genomic region has major effects on several foraging-related behaviors.

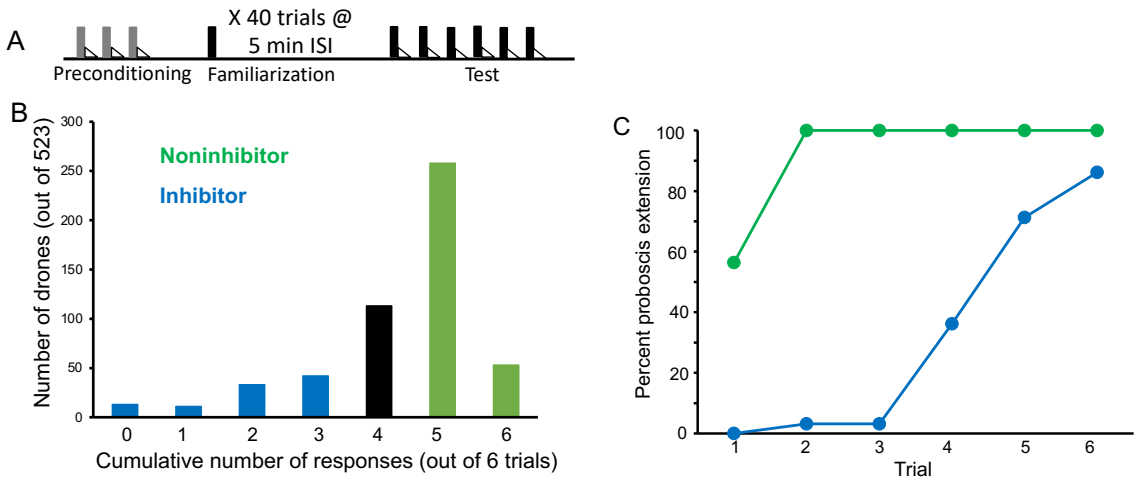

Fig. 1. Evaluation and selection of drones from an F1 queen. **(A)** Drone honey bees were first evaluated by conditioning them over three trials to an odor (A; gray bars) followed by sucrose reinforcement (triangles) in a way that produces robust associative conditioning expressed as odor-induced proboscis extension reflex (PER) (*26*). 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 Supplemental Materials. 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) (*27*) that is expressed in several regions of the honey bee brain (*28-30*). AmTYR1 is most closely related to the insect alpha-2-adrenergic-like octopamine receptors and the vertebrate alpha-2-adrenergic receptors (*31*). 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(*32*), and nurses and foragers differ in AmTRY1 expression(*21*). 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, identified neurons in the honey bee brain (*29, 37*) that 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 (*38, 39*). Finally, octopamine and tyramine affect locomotor activity in the honey bee(*40*).

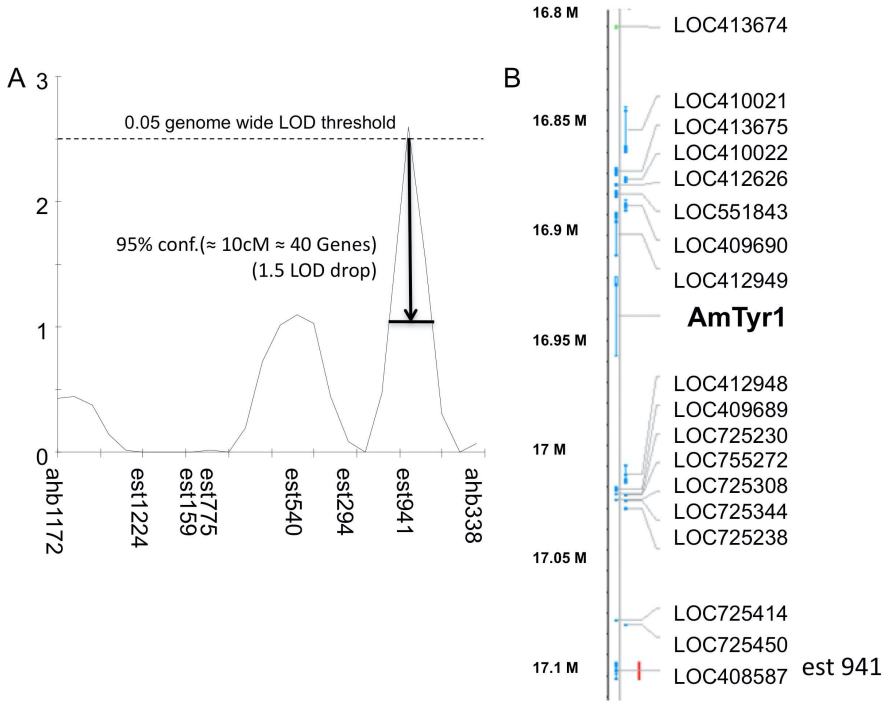

Fig. 2. Single-Nucleotide Polymorphism (SNP) mapping of high and low recombinant drones. (A) Markers from linkage group 1.55 surrounding one significant QTL (est941 with a LOD score of 2.6). **(B)** Partial list of genes within 10 cM of the marker showing the location of *Amtyr1*.

To examine the role of AmTYR1 signaling in latent inhibition, we performed a series of behavioral experiments that involved treatment of honey bees either with the tyramine receptor antagonist yohimbine (*41*) or with a Dicer-substrate small interfering (Dsi) RNA of the receptor (NCBI Reference Sequence: NM_001011594.1) to disrupt translation of mRNA into AmTYR1 (*29, 42*). For these experiments we used unselected worker honey bees from the same background population used for selection studies, which ensured that workers used for behavioral assays would represent a mixture of inhibitor and non-inhibitor phenotypes. Therefore, treatment could increase or decrease the mean level of inhibition in this population. Training involved two phases (Fig. 3A). First, during the 'familiarization' phase honey bees were identically exposed over 40 trials to odor X without reinforcement. Our previous studies have shown that this procedure produces robust latent inhibition. The second 'test' phase involved measurement of latent inhibition. During this phase odor X and a 'novel' odor N were presented on separate trials. Both odors were associated with sucrose reinforcement in a way that produces robust appetitive conditioning (*26*). Latent inhibition would be evident if responses to odor X were lower than the responses to the novel odor N. Injections of yohimbine or siRNA directly into brains occurred either prior to the familiarization phase (Fig. 3A,B) or prior to the test phase (Fig. 3C).

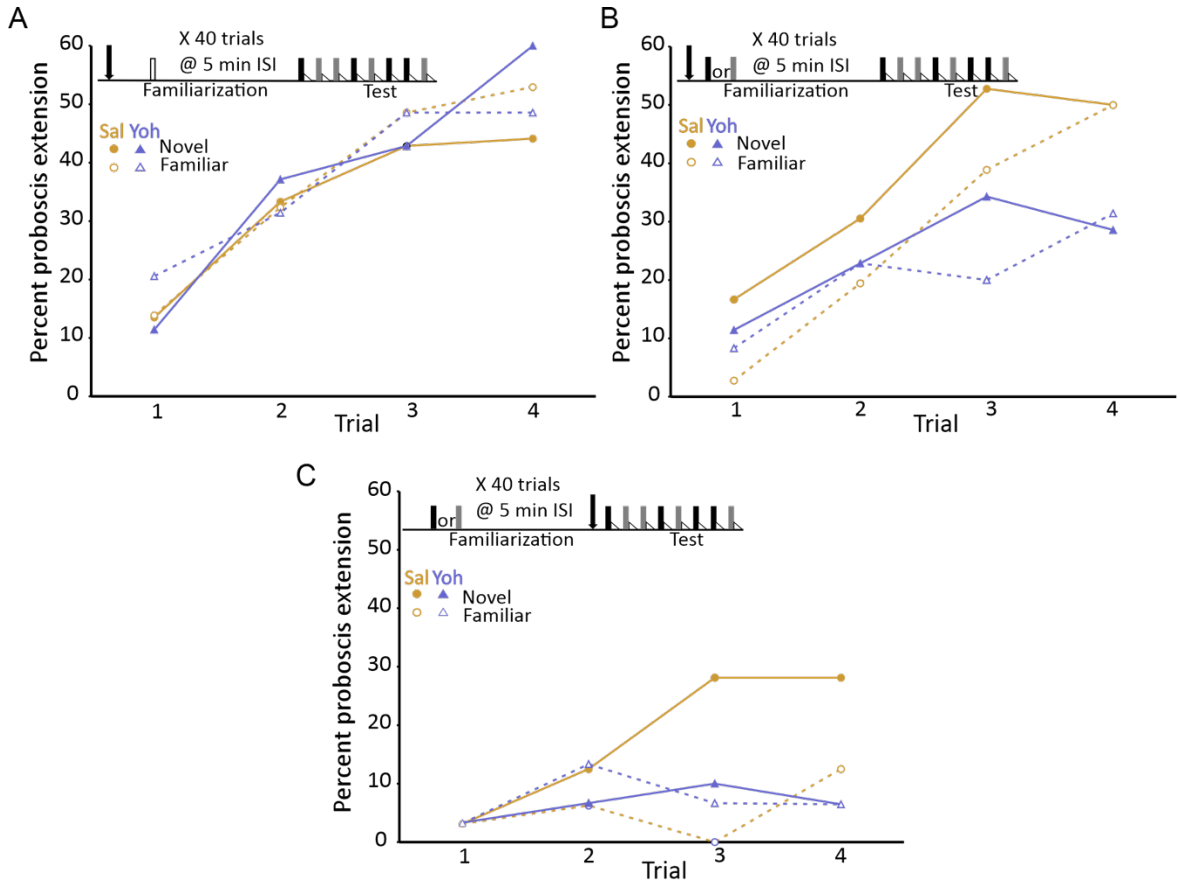

Fig. 3. Blockade of the tyramine receptor with yohimbine increased expression of latent inhibition. (A) Acquisition during the test phase in two injection groups of honey bees familiarized to air as a control procedure to evaluate the effects of yohimbine on excitatory conditioning. The conditioning 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 (blue circles; n = 37 animals) and the other with yohimbine (orange triangles; n=35) prior to preexposure treatment. Because there was no odor presented during familiarization (open box), odors during the test phase were randomly assigned to as novel or familiar. 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 the novel and familiar 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, 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 (blue saline: n=36; orange yohimbine: n=36) in this experiment were familiarized to odor; each of the two odors (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 (*43*).

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 both odors injection groups were equivalent in both. 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, 44-47*).

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 prior to testing (Fig. 3B,C circles). The response to the familiar odor was lower than the response to the novel odor on most trials, including spontaneous responses on the first trial. Injection of yohimbine eliminated the difference in response to the novel and familiar odors. Moreover, the responses to both odors after yohimbine treatment were significantly

lower than, or at least equal to, the response to the familiar odor in the respective saline controls. This pattern could not arise from blockade of excitatory learning about N, because excitatory learning was unaffected in the air preexposure controls (Fig 3A). Instead, the yohimbine-induced pattern was specific to the treatments in which one odor was familiar.

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

Although the results with yohimbine were promising, we decided to disrupt AmTYR1 expression via injection of DsiAmtyr1 in order to provide an independent method to test the role of AmTYR1 in producing latent inhibition (Fig. 4). Yohimbine blocks the receptor, whereas dsiAmTyr1 disrupts production of the receptor protein. Similar outcomes with the two different methods would increase confidence in the result. For the behavioral experiments we used the same procedure as above for yohimbine except that the mixture of three dsiAmtyr1 constructs was injected 20 hours prior to conditioning because of the time frame needed for the DsiRNA to target mRNA. Because of that time frame, and because injection of yohimbine prior to either phase produced equivalent results, we performed injections only prior to familiarization. As a control we used a scrambled sequence of Amtyr1 (DsiScr). Use of DsiScr controls for possible nonspecific effects arising from any aspect of the injection.

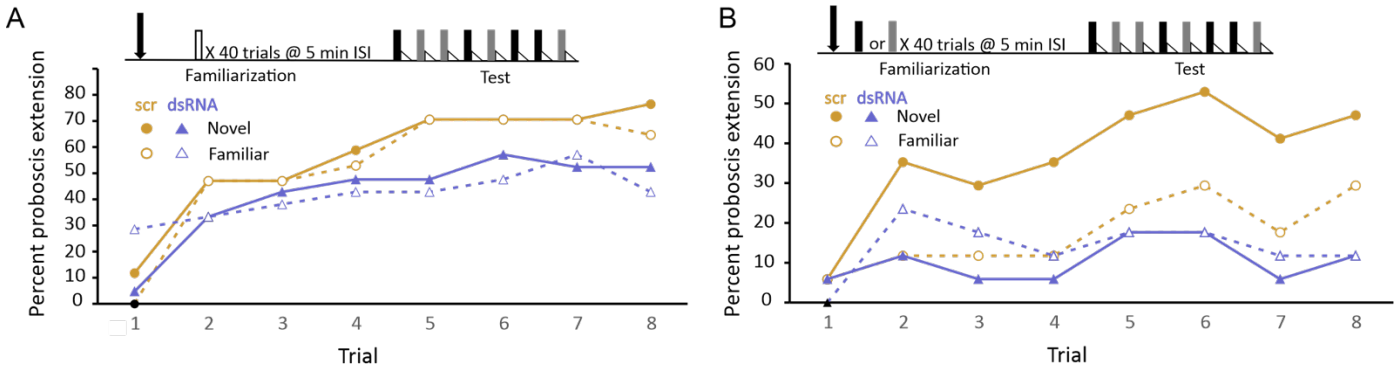

Fig. 4 Disruption of translation of the tyramine receptor by DsiRNA increased expression of latent inhibition. This experiment was identical to that shown in Fig 3A,B, except injections were performed with DsiRNA (arrow) 24 hour prior to behavioral training and testing. The control for this experiment was a scrambled sequence of the *Amtyr1* RNA. **(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 (scr (N=17) DsiRNA (N=13)). The inj*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 DsiAmtyr1 injected animals (0.046+/-0.006) than in Dsiscr injected animals (0.142+/0.028).

Injection of DsiAmtyr1 produced the same effects as yohimbine. 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 DsiAmtyr1 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 DsiAmtyr1 *enhanced* latent inhibition. This result would arise, for example, if AmTYR1 were involved in modulating inhibition to prevent it from becoming too strong.

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(*48*). Familiarization to an odor X caused a mixture of a novel odor N and X to become much more like N. 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, 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(*29*). 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. We used yohimbine in these experiments because of the more rapid onset (minutes versus hours) compared to DsiRNA.

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 could potentially decrease excitatory drive onto inhibitory networks and possibly keep inhibition modulated between two extremes.

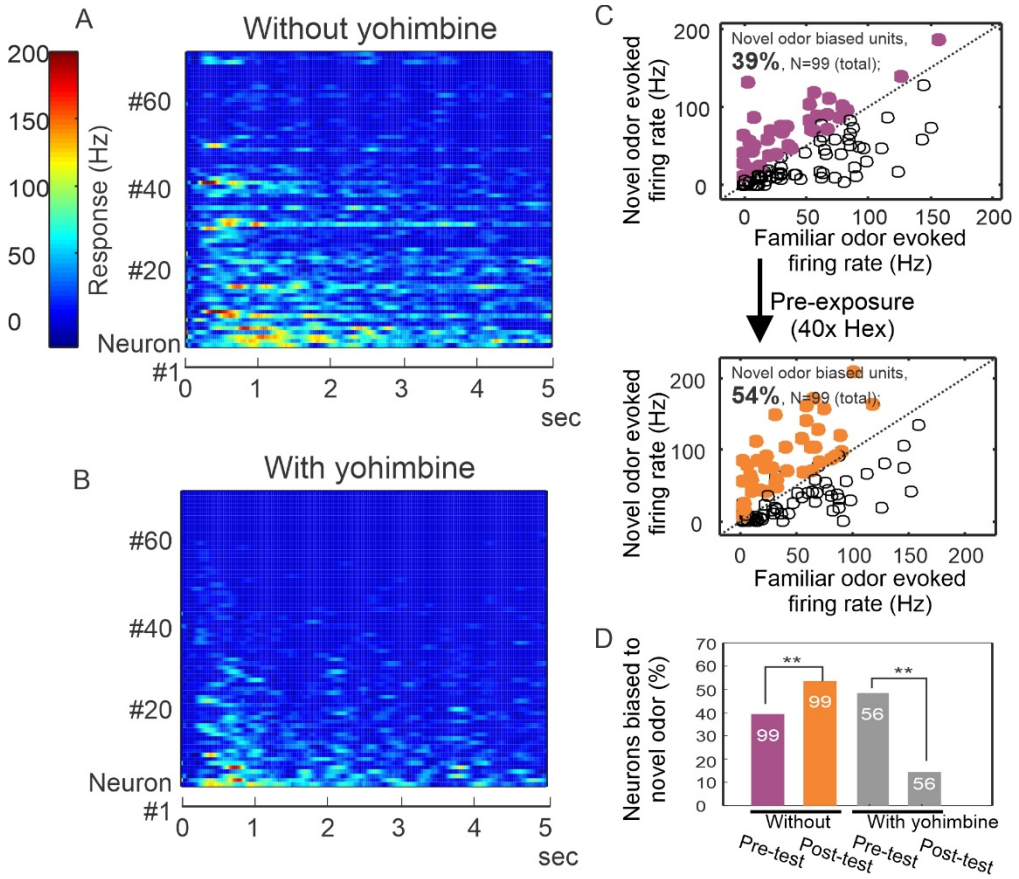

Fig. 5 Yohimbine disrupts latent inhibition in the honeybee antennal lobe. **A-B**. Perfusion of yohimbine solution (50μM in physiological saline) into honey bee head capsule caused antennal lobe units to decrease response magnitude to odor stimuli (2-octanone+1-hexanol) in general. Odor was delivered 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 Hex and Oct, but 39% were biased towards Oct (purple dots above the diagonal line), i.e. showing stronger response to Oct than to Hex. During the familiarization protocol, these units were pre-exposed with Hex 40 times with 1 min interval (arrow down), and were tested again with Hex and Oct 10 min after the last pre-exposing odor stimulation. The test results show 54% of units responded more strongly to Oct (orange dots), which is a novel odor in this protocol. **D**. The 15% increase is statistically significant (*McNemar* test with Yates's correction, d.f.=1, Chi-square=5.939, p=0.015) (asterisks on purple and orange bars, N=99). In contrast, when identical familiarization protocol was used with yohimbine application, such 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=0.0008) (asterisks on grey bars, N=56).

Moreover, yohimbine treatment modified how neurons respond to novelty. Using the same familiarization protocol, we found that 39% of units (N=99) responded more strongly to N before the familiarization to X (Fig.5C, purple dots in upper panel; purple bar in Fig.4D). 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=0.015). Hence familiarization increased bias towards the novel odor in neurons that were more responsive to N to begin with, which is consistent with our earlier results(*48*). In different experiments where yohimbine was applied, the familiarization protocol not only did not increase bias towards N, 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=0.0008), suggesting that yohimbine interrupted this neural manifestation of novelty detection.

Finally, given the implication of *Amtyr1* in latent inhibition, we evaluated whether non-synonymous mutations in the coding sequence might change the functionality of the receptor. We performed a detailed genomic analysis of the 40kb region including the *Amtyr1* gene, a 2kb upstream, and a 0.5kb downstream non-coding region (Fig. 6). SNP frequency in the coding sequence (CDS) was relatively low compared to the genome wide SNP frequency, and all 46 SNPs in the coding regions in any of the sequenced eight individual worker genomes represented synonymous substitutions, i.e., these SNPs do not change the sequence of the encoded protein. Thus, phenotypic differences are not caused by structural changes in the tyramine receptor protein itself. We did, however, find an increased SNP frequency in introns, the up- and downstream non-coding regions and the 3' untranslated region (UTR). These variations might be linked to the changes in the regulation of Am*tyr1* gene expression, e.g. by changes in transcription factor binding sites or the stability of the mRNA, which might eventually be responsible for the observed phenotypic differences. Although, we couldn`t find structural variation in our populations for Amtyr1, the fact that we could map a QTL and the success of both pharmacological interference and genetic knock-downs argues for the involvement of genotypic variation that segregates within our population for the degree of latent inhibition in workers an drones.

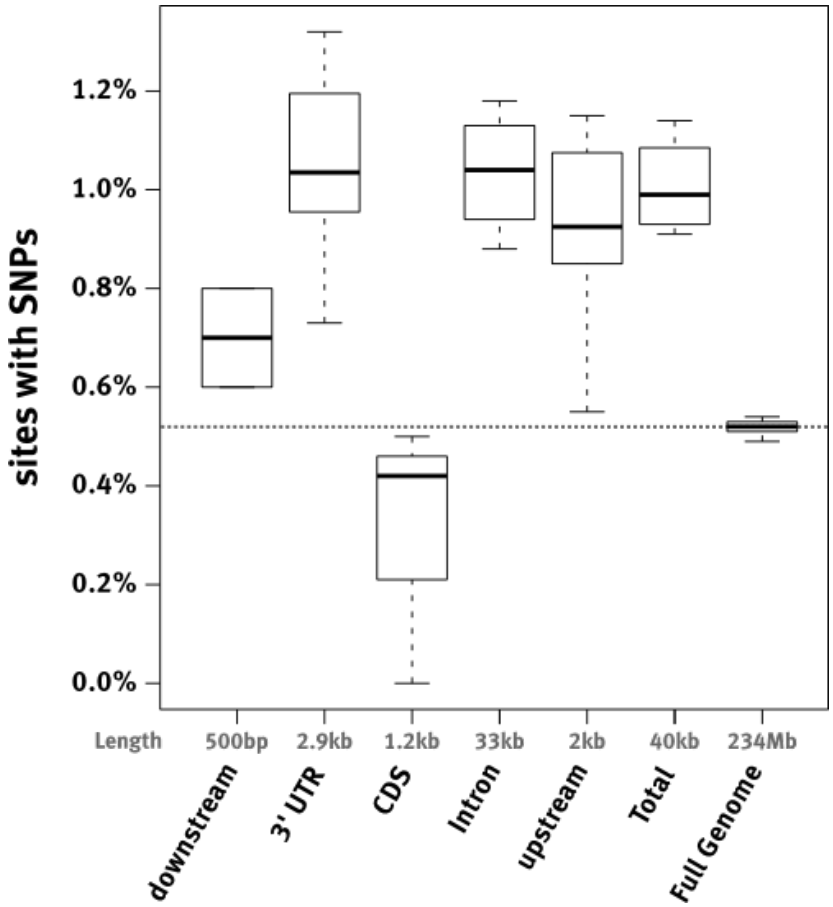

Fig. 6. Single-Nucleotide Polymorphism analysis of different genetic regions of the *Amtyr1* gene. For each type of genetic region, the percentage of sites with SNPs was determined. As baseline, the dotted line indicates the SNP frequency of the full-genome for our eight worker genomes.

A recent report implicated the release of dopamine in driving reward seeking behavior (*49*). 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 (*50*) 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(*50*).

Of the biogenic amines (Figs. 7A,B), only tyramine showed differences between scouts and recruits (see (*50*) for a more complete analysis of these data). Differences in dopamine or serotonin levels were not significant. For the current purpose, we re-analyzed the data to focus on the ratios of tyramine to octopamine and dopamine to serotonin (serotonin was used a reference for dopamine levels in (*49*)). Scouts that showed strong latent inhibition also had significantly lower ratio of tyramine to octopamine than recruits, and that ratio was also lower than scouts and recruits that showed weak latent inhibition (Figs. 7D,E). There were no significant differences in the dopamine to serotonin ratios. Thus, there is an interaction of tyramine and octopamine *production* with behavioral division of foraging labor and expression of latent inhibition. However, dopamine, serotonin and their ratios do not appear to be involved in latent inhibition.

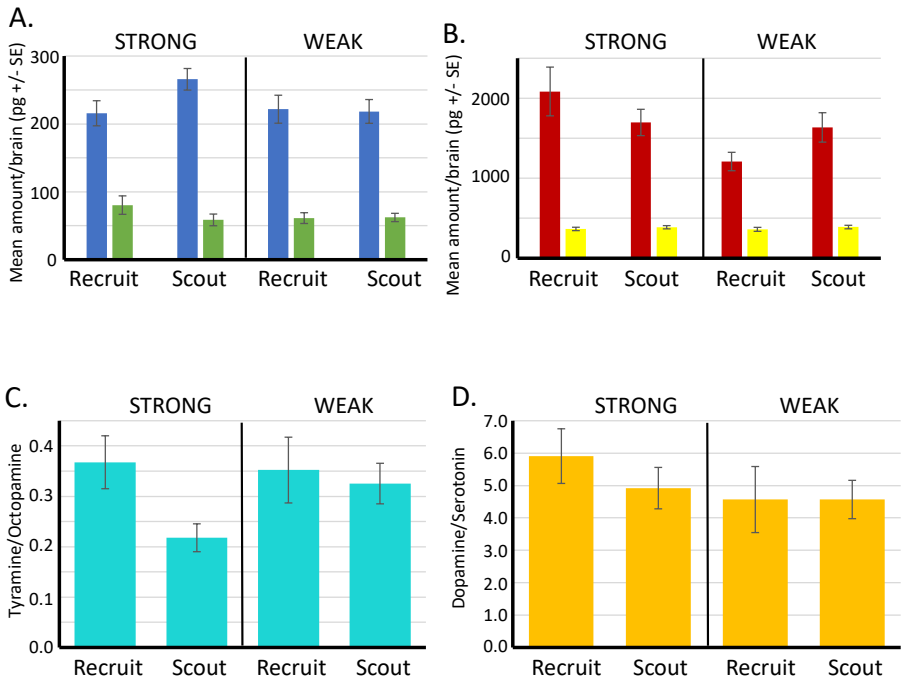

Fig. 7. Biogenic amine levels in individual brains of scout and recruit foragers that expressed strong or weak latent inhibition. A-B. Absolute levels of octopamine (blue) and tyramine (green) and of dopamine (red) and serotonin (yellow) in individual forager

brains. **C.** Ratios of tyramine/octopamine. In foragers that exhibited strong latent inhibition, the ratio was significantly lower in scouts (N=25) than recruits (N=13) (Wilcoxon W=56.0, $p<0.05$). Ratios did not differ 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 of weak groups ($p>0.05$). Sample sizes the same as in C.

Our results have identified the neural underpinnings of a novel and 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 as yet other unidentified genes in the same locus. Nevertheless, our manipulation of AmTYR1 function using both pharmacology and DsiRNA treatments confirm its association with expression of latent inhibition.

However, the precise relationship of *Amtyr1* to latent inhibition is different from what is normally expected from disruption of a gene that underlies a behavior. We expected that disruption of AmTYR1 function would reduce or eliminate latent inhibition; that is, learning about a familiar odor (X) would rise to equal learning about the novel odor. Instead, latent inhibition was strengthened so much so that it appears to generalize even to novel odors. This reduction cannot be explained by nonspecific – e.g. toxic – effects of treatment, because the same treatments did not reduce excitatory conditioning in the absence of familiarization to an odor. Moreover, the same effect was evident using two very different means for disruption of AmTYR1 signaling.

We propose that AmTYR1 modulates inhibition that reduces attention to a familiar odor. AmTYR1 maintains neural inhibition at a set point between the extremes where it becomes too strong (e.g. when AmTYR1 is disrupted) or too weak (AmTYR1 strongly activated). We have shown for honey bees that AmTYR1 is expressed on presynaptic terminals of olfactory sensory neurons in the antennal lobes as well as on presynaptic

terminals of projection neuron axons that terminal in the mushroom body calyces(*29*). 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 excitation of post-synaptic inhibitory processes, which would be consistent with the modulatory role we propose for AmTYR1, particularly if this modification were then embedded in Hebbian-like modification of the synapse. Further investigations can now test this hypothesis.

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 (*38, 39*). 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 would 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 DsiAmtyr1 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, 51*). 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(*51*). 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.

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and analyzed electrophysiological experiments; BHS wrote the paper, integrated feedback from coauthors and supervised the work.

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Data and materials availability: TBD

List of supplementary materials

Materials and methods

Supplementary Materials

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 (*1*). 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 (*2*). 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 (*3*). 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) (*1*) were mated using standard instrumental insemination techniques (*4*) 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 (*1, 5*).

Recombinant drones Male honey bees (drones) were produced from a cross between genetic lines selected for high and low expression of latent inhibition(*1, 6*). Hybrid queens were reared from a cross of a queen from the inhibitor line instrumentally inseminated(*7*) 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 (*3*). 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 2.0 M sucrose solution to the antennae. Drones that extended their proboscis were selected for training.

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

Conditioning protocols

Familiarization: Familiarization to the odor was done as described in (*2*). Restrained bees were placed in individual stalls where a series of valves regulated odor delivery via a programmable logic controller (PLC) (Automation Direct). Hexanol and 2-octanone were used either as pure odorants or diluted to 2.0 M in hexanes with odor treatments counterbalanced across animals. Odor cartridges were made by applying 3.0 µl of odorant onto a piece of filter paper (2.5 x 35 mm) and inserting the filter paper into a 1 ml glass syringe. The odor cartridge was then connected to a valve regulated by the PLC that shunted air through the cartridge for 4 s once the automated sequence was initiated. Odor preexposure in all experiments involved 40 unreinforced presentations of odor for 4 s using a 5 min (Fig 1) or 30 s (Fig 3) intertrial interval (ITI). All odor cartridges were changed for fresh ones after every 10 uses to avoid odor depletion(*8*). The use of pharmacological

treatment necessitated the use of a shorter ITI to avoid having the drug wear off before the end of preexposure. Our previous studies have revealed that latent inhibition is robust over this range of ITIs and odor concentrations (*2*).

Proboscis Extension Response conditioning: All PER learning paradigms used for testing used a 5 min ITI. An acquisition trial consisted of a 4 s presentation of an odor, the conditioned stimulus (CS, black or gray bars), followed by presentation of a 0.4 µl drop of 1.0 M sucrose solution, the unconditioned stimulus (US, triangles in Figs 1, 3 and 4). Three seconds after onset of the CS the US was delivered using a Gilmont® micrometer syringe. The US was initially delivered by gently touching the antennae to elicit proboscis extension and subsequent feeding. Once a bee began to extend its proboscis at the onset of CS 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(*9*). 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 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 (*10*). 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 (*11, 12*). 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).

Pharmacological and dsiAmTyr1 treatments. Yohimbine hydrochloride (Sigma) was diluted to 10^{-4} M in saline (5 mM KCl, 10 mM NaH_2PO_4, pH 7.8). We chose a concentration of yohimbine that has been shown to be effective in our previous study of its effect on honey bee behavior (*13*). One µl of drug or saline alone was injected into the brain through the median ocellus using a Hamilton syringe (Hamilton; Reno, NV). Training began 15 min after injection, as this time has been shown to be effective in other drug studies using the same methodology (*2, 14, 15*).

For dsiRNA studies, we used sequences and protocols developed previously for a study of *Amtyr1* distribution in the brain, which in that study were used to show that the AMTYR antibodies specifically recognized the receptor(*16*). We used a Dicer-substrate small interfering (Dsi) RNA of the *AmTyr1* receptor (NCBI Reference Sequence: NM_001011594.1) to knockdown *AmTyr1* mRNA receptor in the brain. We used the mixture of three dsiAmTyr1 designed by the tool in IDT technology(*16*). As a control we used a scrambled (dsiScr) version of the Amtyr1 sequence. A 138 nanoliter injection of a 100microM mixture of dsiAmTyr1 or dsiScr (Nanoinject 2000) was made into the middle ocellus 18-20 hours before behavioral tests. All injections were done blind so that the investigator doing behavioral tests was not aware of the content of the injection. After the tests brains without optic lobes were dissected out and homogenized each in TRIzol (Invitrogen) (N=27 for bees injected with dsiScr and N=32 for bees injected with dsiAmTyr1). Then, the total mRNA from each injected brain was extracted separately using the manufacturer's protocol for TRIzol method (Invitrogen) Contaminating genomic

DNA was removed using DNA-free™ kit (Ambion, AM1906). RNA quantity and purity was evaluated using a NanoDrop (NanoDrop 2000). Expression of AmTyr1 was quantified using QuantiFAST SYBR Green RT-PCR kit (QIAGEN) on Applied Biosystem 7900 cycler (ASU DNA Facilities) with the protocol provided by the kit for a 384-well plate. The primers for quantitative real-time PCR assays were: AmTyr1_F 5'-GTTCGTCGTATGCTGGTTGC-3', AmTyr1_R 5'- GTAGATGAGCGGGTTGAGGG-3' and for reference gene AmActin_F 5'- TGCCAACACTGTCCTTTCTG-3' AmActin_R 5'-AGAATTGACCCACCAATCCA-3'.

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

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

Sequencing the *Amtyr1* region of the genome. We studied single nucleotide polymorphisms (SNPs) in full-genome sequences of eight *A. mellifera* workers (four high pollen hording and four low pollen hording). For each individual, Illumina short reads were mapped against the *Apis mellifera* genome assembly version 4.5 [Munoz-Torres et al., 2011] using bwa version 0.5.9-r16 [Li and Durbin, 2009]. An average 25x genome coverage per individual allowed the identification of high-quality SNPs in each individual against the reference genome. SNPs were identified with SAMtools version 0.1.17-r973:277 [Li et al., 2009] enforcing a minimum quality score of 20 (base call accuracy ≥ 99%).

Statistical analysis. To analyze the effects in behavioral experiments, we used a generalized linear model with binomial error distribution and logit transformation to perform a logistic regression. The response variable is binomal (0,1). Trial is an ordered variable. We were most interested in testing the hypothesis that injection of yohimbine and dsiRNA before familiarization treatment would impact latent inhibition, so we focused on the interactions between trial, injection, injection time (before pre-exposure or before acquisition), and odor (novel or pre-exposed odor). To explore significant interactions further, we performed a tukey post hoc test using the package emmeans (Lenth 2018). All analyses were performed in R version 4.2.0 using RStudio version 2022.07.1.

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