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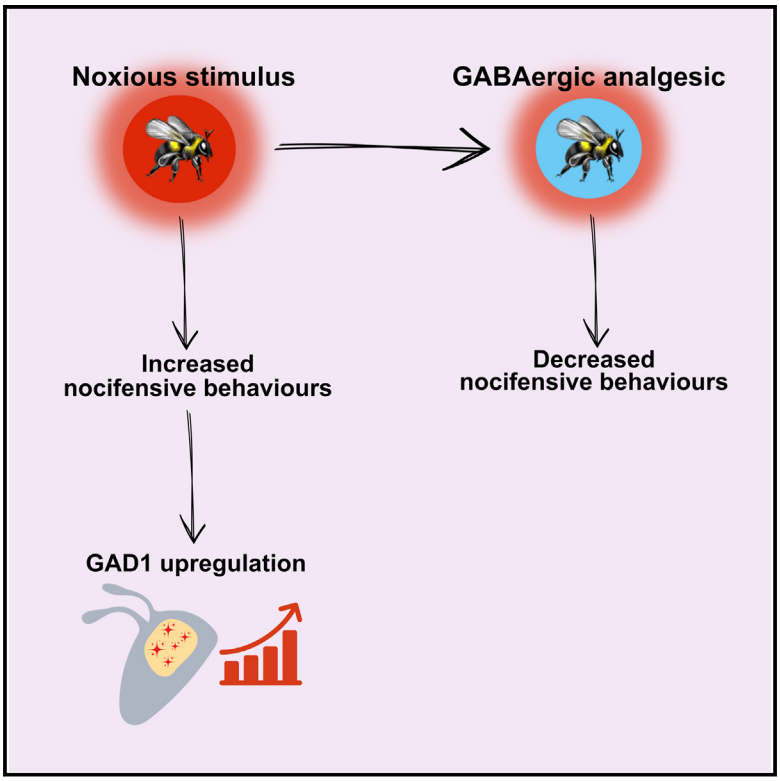
Central GABAergic neuromodulation of nocifensive behaviors in bumble bees

Graphical abstract



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In brief

Zoology; Entomology; Neuroscience

Highlights

- Bumble bees exhibit nocifensive behaviors when exposed to a noxious stimulus
- Gabapentin has an analgesic effect by reducing the nocifensive behavioral pattern
- Noxious stimulation upregulates *GAD1* expression in the brain
- GABA plays a crucial role in modulating nocifensive behaviors in insects

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Central GABAergic neuromodulation of nocifensive behaviors in bumble bees

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SUMMARY

In mammals, nocifensive behaviors are modulated by neuroactive compounds indicating a complex pain-processing system. This study aimed to assess the role of gamma-aminobutyric acid (GABA) in modulating nocifensive behaviors in the bumble bee *Bombus terrestris* to better understand how pain-like behaviors are regulated in insects. Results showed that oral administration of gabapentin (0.24 mg/mL), a GABAergic analgesic acting at the central level, reduced heat sensitivity, making bees more likely to pass through a tunnel heated to 50°C, and suppressed their nocifensive behaviors. Gene expression analysis revealed a significant increase in brain expression of GAD1 in response to noxious stimulation. These findings indicate that GABA plays a key role in modulating nocifensive behaviors in insects, similar to its role in mammals, suggesting that insects may possess mechanisms for pain modulation that go beyond simple peripheral responses.

INTRODUCTION

Across animal taxa, nociceptors—peripherally localized neurons sensitive to noxious stimuli—mediate the sensory process of nociception.[1–3] This mechanism allows animals to respond appropriately to harmful stimuli, ensuring their survival.[4] Nociception—the neural process of encoding and processing noxious stimuli—manifests as observable responses to stimuli, i.e., nocifensive behaviors.[5] In vertebrates, nociceptors are characterized by free nerve endings beneath the epidermis, enabling integrated nocifensive responses, including rapid reflexes and more complex behaviors, such as vocalizations, avoidance, and protections.[6–8] Analogous structures have also been identified in insects, such as class IV multidendritic neurons in *Drosophila melanogaster*, which are responsible for responses to noxious thermal, mechanical, and chemical stimuli.[9,10] Genetic manipulation of these neurons eliminates the characteristic rolling behavior, a crucial defensive response.[11] Studies on other species, including *Manduca sexta*[12] larvae and adult cockroaches,[13] suggest an evolutionary conservation of these nociceptive mechanisms. Additionally, ion channels, such as transient receptor potential (TRP) and degenerin/epithelial sodium channels (DEG/ENaC), which are key features of vertebrate nociceptive neurons, are present in many species, including flies, beetles, moths, and cockroaches, further confirming an ancient evolutionary origin.[14,15] In *Hymenoptera*, the HsTRPA channel replaces TRPA1, responding to noxious thermal and chemical stimuli.[16] For example, in *Apis mellifera*, the inhibition of HsTRPA blocks the nocifensive sting response.[17] However, nocifensive behaviors in insects are generally considered more reflexive,[18] although they can vary depending on the stimulus and may involve context-dependent modulation,[19,20] and little is known about the broader mechanisms of nociception and behavioral responses in insects.

In vertebrates, particularly mammals, nocifensive behaviors can be modulated by neurobiological mechanisms that extend beyond the peripheral system, and involve the central nervous system, where the subjective negative experience of pain originates.[1] An organism capable of experiencing pain, defined as the conscious perception of harm or discomfort beyond mere nociception, is considered sentient, that is, it has the capacity for subjective experience, including emotional responses to stimuli. The relationship between nociceptive behavior and the subjective experience of pain in non-human animals, especially invertebrates such as insects, is not as well established as in humans or other mammals and remains controversial. Despite a growing body of research demonstrating complex behaviors and neurobiological pathways in insects that may indicate the capacity to experience pain,[19] a thorough understanding of how nocifensive behaviors are influenced by the central nervous system in these animals remains largely unexplored.[21]

To understand the mechanisms underlying the experience of pain in animals, it is important to identify how modulation of nocifensive behaviors occurs. Evidence for the existence of pain beyond nociception is provided when the animal's behavioral response to a noxious stimulus is modulated by chemical compounds that inhibit the transmission of nociceptive signals and

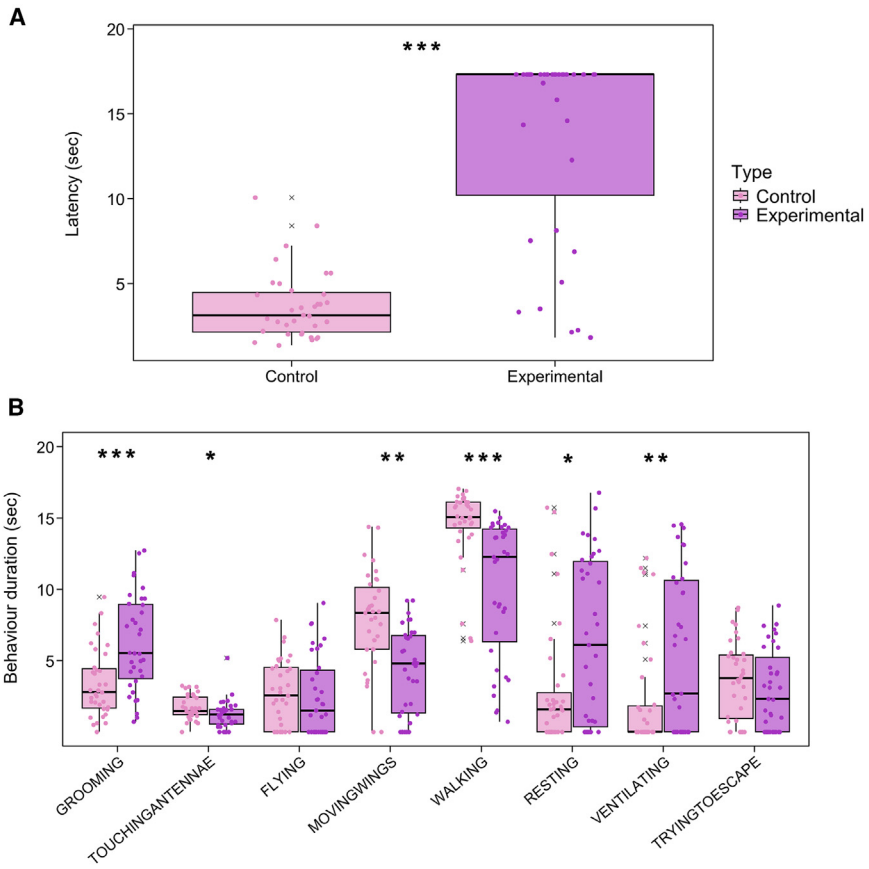

Figure 1. Identification and quantification of nocifensive behaviors

(A) Median ± interquartile range of latency time taken by the bumble bees in the *Control* (pink) and *Experimental* (purple) conditions to enter the tunnel; Wilcoxon signed rank test.

(B) Median ± interquartile range of the time spent performing the 8 behaviors in the *Control* (pink) and *Experimental* (purple) conditions; Wilcoxon signed rank test. Data in both panels are square root-transformed. Crosses indicate values outside this range (boxplot outliers). Dots represent individual bees.

$p < 0.001$ are indicated by "***"; $0.001 < p < 0.01$ are indicated by "**"; $0.01 < p < 0.05$ are indicated by "*".

act as pain modulators.[19,22] The modulation of nociceptive signals can occur through the activation of endogenous neurotransmitter systems or the administration of exogenous analgesics which can interfere with both ascending and descending pain pathways.[22,23]

In mammals, one of the key endogenous neurotransmitters involved in pain modulation is gamma-aminobutyric acid (GABA).[24] Furthermore, recent evidence, reviewed by Gibbons, Crump et al., 2022, points to a potential role for GABA in nociceptive modulation in insects. In particular, there is evidence that GABA may be involved in modulating responses to noxious heat in fruit flies.[23,25–27]

This research aims to deepen our understanding of the underlying neurotransmitter modulation of nocifensive behavioral response in insects with a particular focus on the role of GABA. Specifically, we tested whether exposure to a noxious stimulus, i.e., a tunnel heated to 50°C, elicits nocifensive behaviors in the bumble bee *Bombus terrestris* and, secondly, we combined behavioral observations with pharmacological manipulation to assess whether the elicited nocifensive behaviors can be modulated by exogenous administration of a GABAergic analgesic, i.e., gabapentin. If drugs are able to modify the nocifensive behaviors of these animals, it would suggest that bumble bees can modulate their responses to a noxious stimulus using neuroactive compounds. This would increase confidence in the presence of analgesia in Hymenoptera[19] and provide stronger evidence supporting their ability to perceive pain.

Finally, to explore the potential neurobiological changes underlying nocifensive behaviors and to target the neurotransmitter and signaling pathway involved, we used quantitative reverse-transcription polymerase chain reaction (RT-qPCR), a molecular technique for amplifying and quantifying specific target genes, to examine the expression in the brain of five key GABA-related genes, (i.e., ABAT, CaMKII, GABAr, GAD1, and SYN).[28] This allowed us to investigate the expression levels of these genes in response to a noxious stimulus and provide further evidence on the molecular mechanisms through which GABA may influence pain perception and response in insects, contributing to a more comprehensive understanding of their ability to experience pain.

RESULTS

Experiment 1: Identification and quantification of nocifensive behaviors

The latency time taken by the bumble bees to enter the tunnel in the control condition (tunnel off; hereafter *Control*) was significantly different from the time taken by the same individuals to enter the tunnel in the experimental condition (tunnel heated at 50°C; hereafter *Experimental*) (Wilcoxon signed rank test, $p = 9.3e\text{-}07$), (Figure 1A).

The linear model (LM) model ($R^2 = 0.7627$, adjusted $R^2 = 0.7619$, $F = 895.2$, $p < 2.2e\text{-}16$) showed that the general effect of the experimental condition (*type*) on *duration* of behaviors was not significant ($\beta = 0.1300$, $SE = 0.2087$, $p = 0.533$). However, introducing interaction terms between *type* and *behavior* revealed significant differences for specific behaviors ($R^2 = 0.8745$, adjusted $R^2 = 0.8708$, $F = 236.6$, $p < 2.2e\text{-}16$). Wilcoxon signed rank test confirmed that the bumble bees in *Control* and *Experimental* significantly differ in time in the following behaviors: grooming ($V = 103$, $p = 0.0003$), touching antennae ($V = 447$, $p = 0.03$), moving wings ($V = 507$, $p = 0.001$), walking ($V = 558$, $p < 0.001$), resting

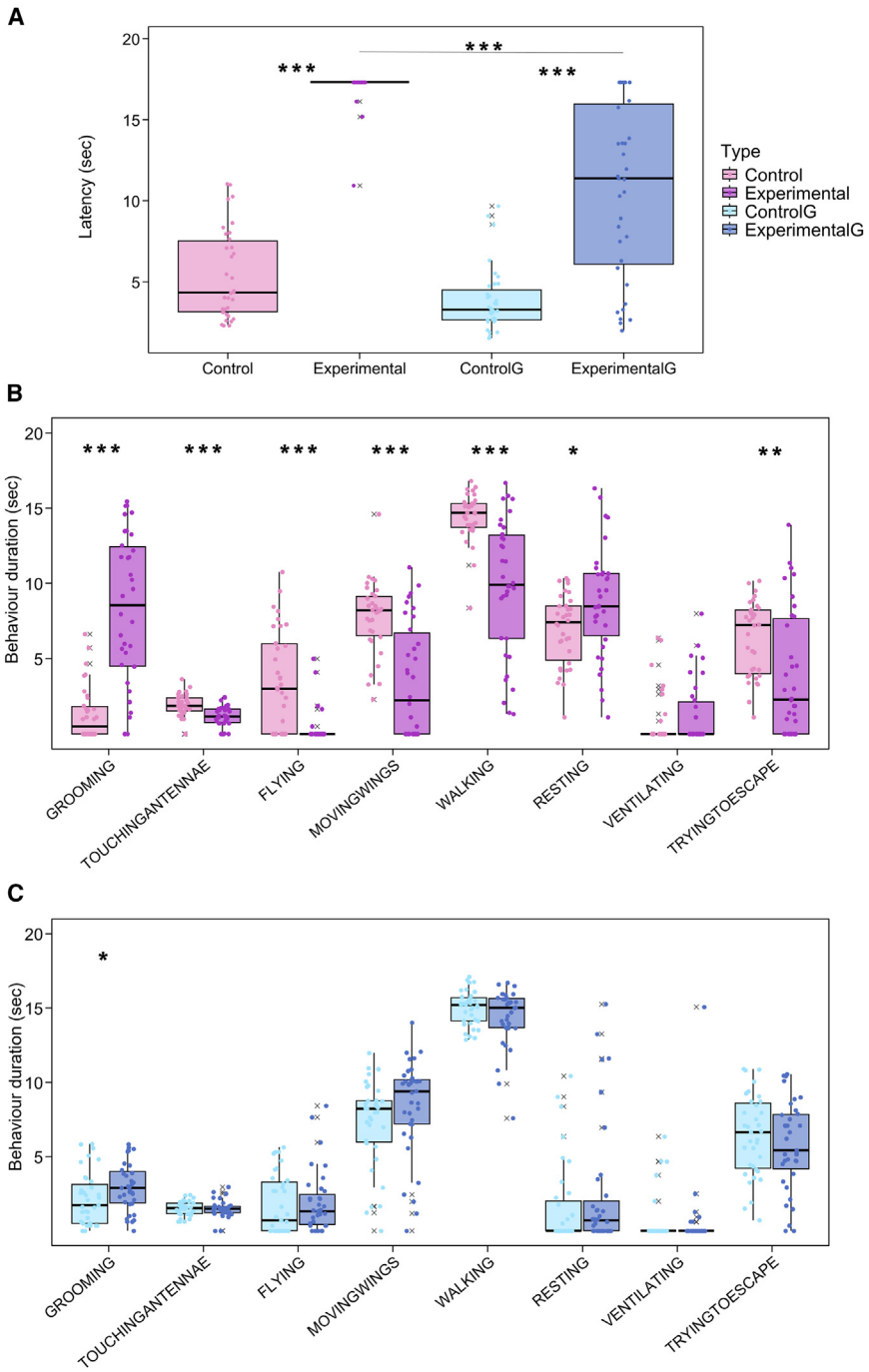

Figure 2. Effects of drug manipulation on nocifensive behaviors

(A) Median ± interquartile range of latency time taken by the bumble bees in the *Control* (pink), *Experimental* (purple), *ControlG* (light-blue), and *ExperimentalG* (blue) conditions to enter the tunnel; Kruskal-Wallis test and post-hoc Dunn test.

(B) Median ± interquartile range of the time spent performing the 8 behaviors in the *Control* (pink) and *Experimental* (purple) conditions; Wilcoxon rank-sum test.

(C) Median ± interquartile range of the time spent performing the 8 behaviors in the *ControlG* (light-blue) and *ExperimentalG* (blue) conditions; Wilcoxon rank-sum test. Data in all panels are square root-transformed. Crosses indicate values outside this range (boxplot outliers). Dots represent individual bees.

$p < 0.001$ are indicated by "***"; $0.001 < p < 0.01$ are indicated by "**"; $0.01 < p < 0.05$ are indicated by "*".

(V = 108, $p = 0.02$), and ventilating (V = 59, $p = 0.009$) but not in flying (V = 293, $p = 0.3832$) and trying to escape (V = 347, $p = 0.2383$), (Figure 1B).

Experiment 2: Effects of drug manipulation on nocifensive behaviors

Gabapentin effect on nocifensive behavioral pattern

Kruskal-Wallis showed differences in latency duration between the four groups (the two treated with sucrose solution only: *Control*, *Experimental*, and the two treated with sucrose solution + gabapentin: *ControlG*, *ExperimentalG*) in Exp 2 (χ^2 = 86.271, df = 3, $p < 2.2e-16$). The post-hoc Dunn test performed with Bonferroni correction identified which pairs of groups exhibited significant differences (*Control* vs. *Experimental*, $p = 1.05e-12$, p adjusted <0.001; *ControlG* vs. *ExperimentalG*, $p = 4.69e-06$, p adjusted <0.001; *Experimental* vs. *ExperimentalG*, $p = 5.97e-05$, p adjusted <0.001) (Figure 2A).

In the groups treated with sucrose solution only (*Control* and *Experimental*), the LM analysis (R^2 = 0.8337, Adjusted R^2 = 0.8330, F = 1356, $p < 2.2e-16$) showed that the general effect of the experimental condition (*type*) on *duration* was not significant ($\beta = -0.196$, SE = 0.194, $p = 0.31$). However, the interactions between *type* and *behavior* showed significant results (R^2 = 0.9268, adjusted R^2 = 0.9245, F = 416.7, $p < 2.2e-16$). Wilcoxon rank-sum test confirmed that the bumble bees in *Control* and *Experimental* significantly differ in time in the following behaviors: grooming (W = 135.5, $p = 3.985e-08$), touching antennae (W = 885.5, $p = 0.0001652$), flying (W = 899, $p = 8.645e-06$), moving wings (W = 926, $p = 1.861e-05$), walking (W = 957, $p = 1.065e-06$), resting (W = 394, $p = 0.02373$), trying to escape (W = 813, $p = 0.003967$) but not in ventilating (W = 524, $p = 0.4069$), (Figure 2B).

In the groups treated with sucrose solution + gabapentin (*ControlG* and *ExperimentalG*), the LM analysis ($R^2 = 0.7436$, adjusted $R^2 = 0.7427$, F = 807.8, $p < 2.2e-16$) indicated that the general effect of the experimental condition (*type*) on *duration* was not

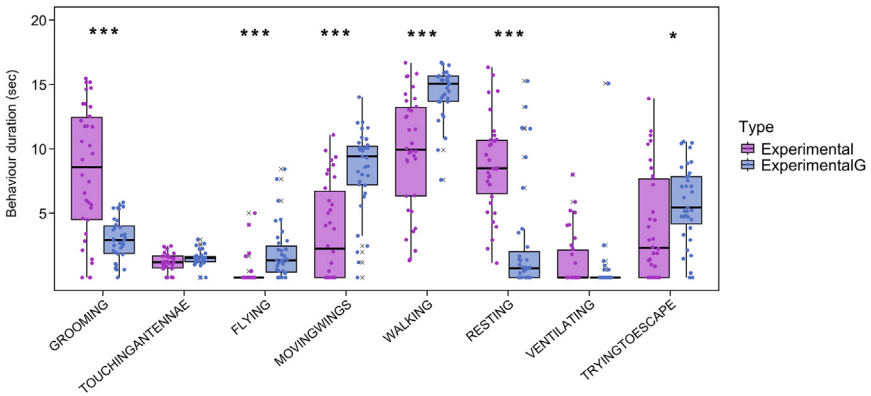

Figure 3. Gabapentin effect on noxious stimulation

Median ± interquartile range of the time spent performing the 8 behaviors in the *Experimental* (purple) and *Experimental*G (blue) conditions; Wilcoxon rank-sum test. Data are square root-transformed. Crosses indicate values outside this range (boxplot outliers). Dots represent individual bees.

$p < 0.001$ are indicated by "***" and $0.01 < p < 0.05$ are indicated by "*".

significant ($\beta = -0.122$, SE = 0.224, $p = 0.586$). The introduction of interaction between *type* and *behavior* revealed significant differences for specific behaviors (R^2: 0.9153, adjusted R^2: 0.9128; F: 366.6, $p < 2.2e\text{-}16$). Wilcoxon rank-sum test further confirmed that bumble bees in the *ControlG* and *ExperimentalG* groups differed significantly in grooming time (W = 427.5, p value = 0.0302), while no significant differences were found for other behaviors: touching antennae (W = 644.5, $p = 0.7114$), flying (W = 207, $p = 0.1921$), moving wings (W = 452.5, $p = 0.061$), walking (W = 681, $p = 0.4268$), resting (W = 494.5, $p = 0.1376$), trying to escape (W = 682, $p = 0.4177$), ventilating (W = 604, $p = 0.8821$), Figure 2C).

Key finding: Gabapentin effect on noxious stimulation

The Kruskal-Wallis test revealed significant differences in latency duration between the four groups in Experiment 2 (see aforementioned). A post-hoc Dunn test with Bonferroni correction specifically showed a significant difference between the *Experimental* and *ExperimentalG* groups ($p = 5.97e\text{-}05$, p adjusted <0.001) (Figure 2A).

The LM analysis ($R^2 = 0.7782$, adjusted $R^2 = 0.7774$, F = 963.3, $p < 2.2e\text{-}16$) indicated that the general effect of the drug in the experimental condition (noxious stimulation) on *duration* was significant ($\beta = 0.464$, SE = 0.219, $p = 0.034$). The inclusion of the interaction between *Drug (*2 levels: *DrugNone* and *DrugGabapentin)* and *behavior* revealed significant differences for several behaviors (R^2: 0.9006, adjusted R^2: 0.8976; F: 303, $p < 2.2e\text{-}16$). To further explore the effect of the drug on individual behaviors, the Wilcoxon rank-sum test was used to assess differences between *Experimental* and *ExperimentalG*. Significant effects were observed for the following behaviors: grooming (W = 958.5, $p = 1.317e\text{-}05$), flying (W = 201, $p = 4.087e\text{-}07$), moving wings (W = 196.5, $p = 1.574e\text{-}06$), walking (W = 208, $p = 1.112e\text{-}06$), resting (W = 1030, $p = 1.633e\text{-}07$) and trying to escape (W = 406, $p = 0.02331$). No significant differences were found for the following behaviors: touching antennae (W = 450.5, $p = 0.08381$) and ventilating (W = 702.5, $p = 0.08951$) (Figure 3).

Experiment 3: Quantification of GABA genes expression

The t test showed a significant difference in the relative gene expression between the *Control* and *Experimental* groups in GAD1 ($t = -2.5698$, df = 10, $p = 0.0279$) but not in ABAT ($t = -0.015501$, df = 10, $p = 0.9879$), CaMKII ($t = 1.2243$, df = 10, $p = 0.2489$), GABAr ($t = 1.1635$, df = 10, $p = 0.2716$), and SYN ($t = 1.7934$, df = 10, $p = 0.1032$) (Figure 4).

DISCUSSION

In this study, we quantified nocifensive behaviors in bumble bees (Table 1) and investigated whether they were modulated by the administration of a GABAergic drug. Additionally, we explored whether nocifensive behaviors were associated with changes in the expression of key GABA-related genes. Our results from Experiment 1 showed that when bees experience a noxious stimulus (Figure 5), they become more reluctant to cross the *nociceptive tunnel*, thus significantly increasing their latency time compared to the control condition. In addition, we observed several nocifensive behaviors after passing through the *nociceptive tunnel*, such as increased grooming, resting, and ventilating activities, and decreased locomotor activities such as walking, moving wings, and touching antennae. The pharmacological test (Experiment 2) revealed that the behavioral pattern observed in Experiment 1 was suppressed when gabapentin, a GABAergic analgesic active at the central level,[29,30] was administered. The genetic analysis (Experiment 3) showed that nociceptive stimulation was linked to the activation of the GABAergic system, evidenced by an increase in the gene expression of GABA-synthesizing enzyme, GAD1.[31]

In Experiment 1, our first significant finding was an increased latency for bees to enter the tunnel in the experimental condition (*Experimental*) compared to the control condition (*Control*). This suggests that the bees perceive the noxious stimulus and are less willing to cross the tunnel. In Experiment 2, in the experimental condition we observed a significant reduction in latency time for bees treated with the analgesic (*ExpG*) compared to the bees fed only with sucrose (*Exp)*. This result highlights the efficacy of gabapentin in reducing sensitivity to the noxious stimulus, although such reduction was not sufficient to completely prevent heat avoidance, as indicated by the maintenance of a significant difference in latency times compared to the control bees (with and without gabapentin, i.e., *Control* and *ControlG*).

These findings, from initial heat avoidance to its reduction by gabapentin, are consistent with the research by Manev and Dimitrijevic (2004) in *Drosophila*, where a spiral tube with a heat

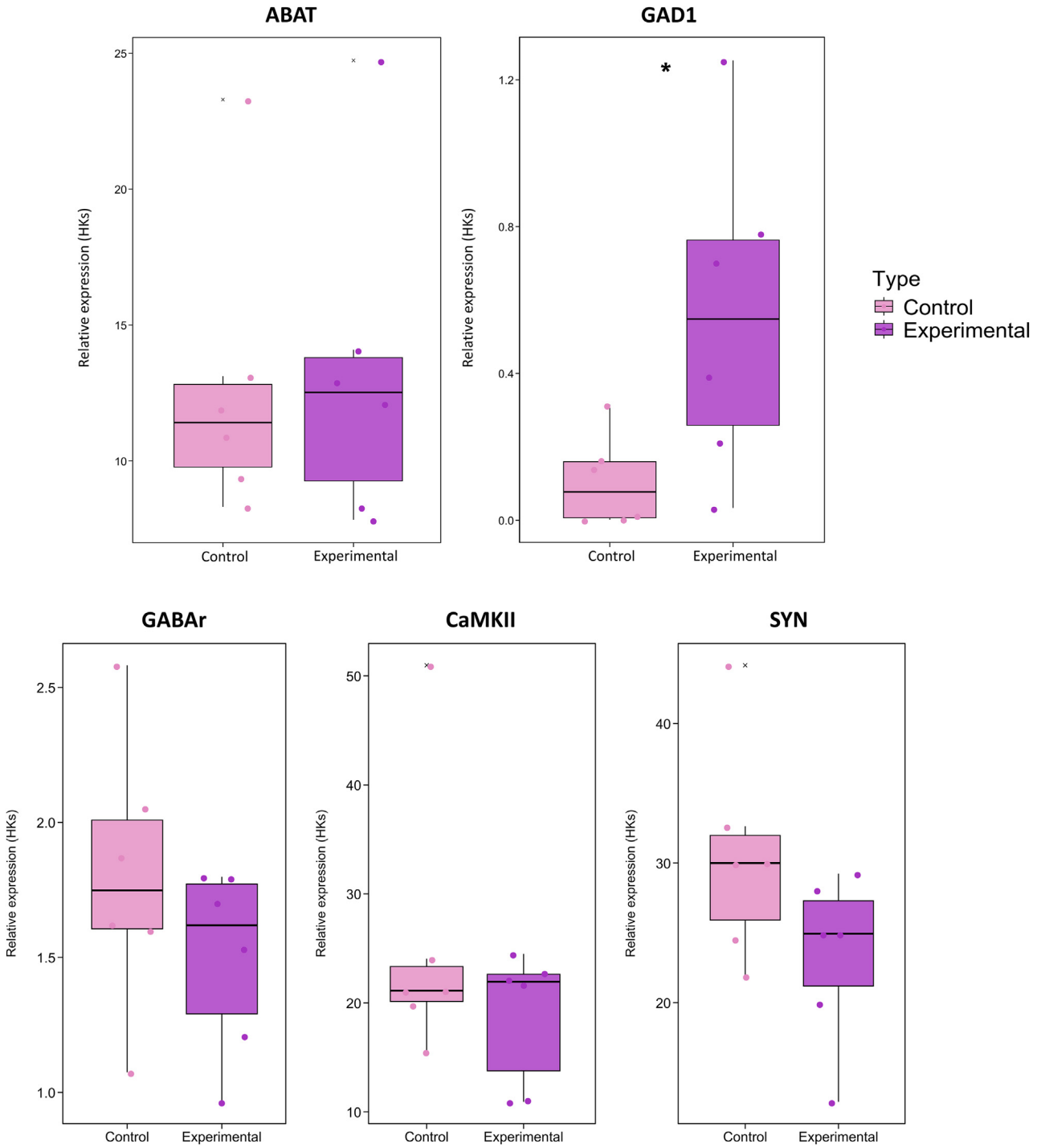

Figure 4. Quantification of GABA genes expression

Median ± interquartile range of relative gene expression of GAD1, ABAT, GABAr, CaMKII, and SYN in bumble bee brain in *Control* (pink) and *Experimental* (purple); t test. Crosses indicate values outside this range (boxplot outliers). Dots represent individual bees.
$0.01 < p < 0.05$ are indicated by "*".

barrier was used to study heat avoidance. Control flies showed an innate aversion to heat, avoiding passage through a 42°C and 60°C tunnel. Conversely, flies treated with the GABA-B receptor agonist 3-APMPA showed altered heat avoidance at 42°C and 60°C and crossed the barrier. This indicates the potential of 3-APMPA to modulate sensitivity to heat stimuli,

Table 1. Ethogram

BEHAVIOR	DEFINITION
Grooming	Bee scratches with its legs another part of the body in a repeated back and forth motion
Touching antennae	Bee touches antennae or mouth parts with its legs for a few seconds without scratching
Walking	Bee is moving around the observation area by stepping
Resting	Bee does not move (freezing)
Ventilating	Bee is expanding and contracting its abdomen, displaying a visible abdominal pulsation
Trying to escape	Bee tries to get out of the observation area, biting corners or digging frenetically
Flying	Bee is moving around the observation area by using its wings
Moving wings	Bee moves its wings without flying, usually performed during walking

Ethogram describing the eight behaviors performed by the bumble bees during the 5-min observation period after passaging through the tunnel.

demonstrating its analgesic activity. It is well established in the literature that activation of GABA-B receptors mediates analgesia in vertebrates[32–34] and recent evidence suggests a similar role in *Drosophila*.[27,31] Similarly to GABA-B receptor agonists, such as baclofen, gabapentin has been shown to induce anti-allodynic effects in rats,[35] suggesting that both drugs may have the same applications in the treatment of pain, although the precise mechanism by which gabapentin exerts its effects remains less clear.[36] Despite being a GABA analog, gabapentin has limited affinity for GABA-A or GABA-B receptors,[37] but it increases GABAergic neurotransmission and GABA levels in the brain.[38–40] Research suggests that its analgesic effect is mediated by the interaction with the $\alpha 2$-δ subunit of voltage-gated calcium channels (VGCCs), in particular with Cav $\alpha 2$-δ located at synapses.[41] As in vertebrates, recent research has shown that treatment with gabapentin prior to nerve injury successfully prevents thermal allodynia in *Drosophila* and also reduces neuropathic leg amputation pain by acting on the $\alpha 2\delta$ subunit of VGCCs.[25] Our latency result in *ExpG* aligns with those observed in *Drosophila*, highlighting the efficacy of gabapentin in modulating pain.

Focusing on the behavioral data of Experiment 1, passage through the nociceptive tunnel induced nocifensive behaviors in the tested bumble bees: compared to the control condition, some behaviors increased in duration and others decreased. In line with the nocifensive behaviors found in vertebrates and used as markers of pain experience,[42,43] we observed an increase in grooming, resting and ventilating, and a decrease in locomotor activities, such as walking, moving wings, and touching antennae. Although a nocifensive ethogram has not yet been systematically described for insects, they are known to exhibit nocifensive behaviors in response to noxious stimuli, such as withdrawal from intense heat sources or chemical irritants.[21] An example is the corkscrew-like nocifensive rolling observed in *Drosophila* larvae in response to noxious temperatures, which acts as a protective escape behavior.[11] Furthermore, an increase in self-grooming in the injured area was observed in *Manduca sexta*[44] and in *Periplaneta americana*.[45] However, these observations have never been quantified. Only recently, a study on *Bombus terrestris* demonstrated and quantified an increase in grooming in the area of the noxious stimulation following injury by a heat probe at 65°C.[46] This increase in grooming, interpreted as an indicator of discomfort and pain in vertebrates, may indicate a similar response in insects.

In Experiment 2, in the *Control* and *Experimental* groups treated with sucrose solution, the results confirmed the nocifensive behavioral pattern observed in Experiment 1. The nociceptive stimulation again induced an increase in grooming and resting, while behaviors like walking, moving wings, and touching antennae decreased. However, compared to Experiment 1, there was also a significant reduction in flying and trying to escape behaviors, while ventilating showed an increasing trend, though not statistically significant. These changes could be attributed to the 1-h waiting period following sucrose administration—a step that was absent in Experiment 1—which may have introduced additional stress. In the comparison between the groups treated with gabapentin (*ControlG* and *ExperimentalG*), the nocifensive pattern was suppressed. No significant differences were found between the control and experimental groups, except for grooming, where the drug was not able to completely suppress this behavior as effect of the nociceptive stimulation. This suggests that gabapentin effectively reduced perception of the stimulus as noxious, although it did not completely abolish the grooming behavior, indicating that the drug reduces sensitivity to pain but does not entirely eliminate the associated behavioral response. Moreover, all bumble bees treated with gabapentin survived during the consecutive three days following the administration. This finding supports the idea that gabapentin does not exert toxic effects in the tested dosage, further reinforcing its potential as a selective analgesic in bees.

By focusing specifically on the bees exposed to noxious stimulation (*Experimental* and *ExperimentalG*), the effect of the analgesic can be more clearly observed. Drug treatment with gabapentin decreased nociceptive-related behaviors (i.e., grooming and resting), and increased activity-related behaviors (i.e., flying, moving wings, walking, and trying to escape) in bumble bees passing through the nociceptive tunnel. This change in behavior indicates an overall reduction in sensitivity to noxious stimulus, suggesting that gabapentin acts as an effective central analgesic. Our behavioral results are consistent with observations in *Drosophila* where gabapentin was found to reduce nociceptive behaviors such as the capsaicin response and larval rolling.[23] It is worth notice that a common limitation of studies investigating the effects of analgesics on insects is the fact that the reduction in overall mobility and reactivity is often used to justify the reduction in nociceptive-related behaviors.[19] However, our results show that the analgesic we used only affects nociceptive-related behaviors, without having a general sedative effect. Consequently, the reduction in nociceptive-related behaviors observed after gabapentin use cannot be attributed to a general reduction in insect reactivity or mobility. It is known that gabapentin specifically targets the central nervous system in humans,[29,30,47]

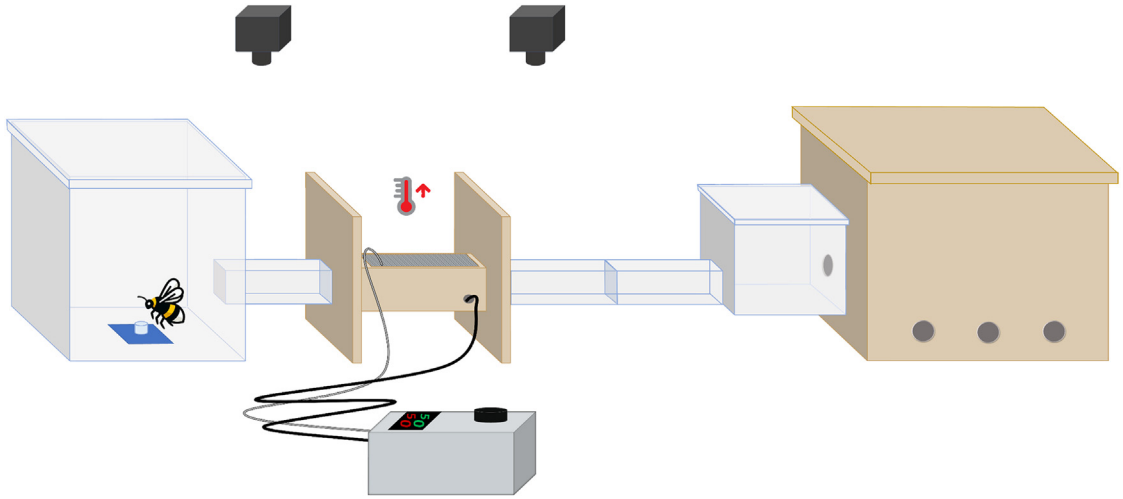

Figure 5. Experimental set-up

Graphic illustrations of the experimental set-up. Bumble bee is represented in a feeding position in the foraging area. We refer to the tunnel between the foraging area and the nociceptive tunnel as "latency observation area" (recorded by the first camera), and to the tunnel after the nociceptive tunnel as "behavioral observation area" (recorded by the second camera).

non-human vertebrates such as rats[48] and even insects.[25] Therefore, if the drug affects nociceptive-related behaviors in bees, it implies that the brain plays a role in modulating this analgesic effect, rather than being a purely peripheral response at the nociceptor level.

Experiment 3 investigated the neurobiological mechanisms underlying nocifensive behaviors by analyzing the expression of GABA-related genes using RT-qPCR (Table 2). The results showed a significant upregulation of GAD1 gene expression in the *Experimental* group, indicating activation of GABAergic pathways in bees exposed to a noxious stimulus. In contrast, no significant changes in expression were observed for other GABA-related genes, such as ABAT, CaMKII, GABAr, and SYN. In insects, GAD1 is analogous to GAD67 in mammals, encoding the enzyme glutamate decarboxylase, which is essential for GABA synthesis.[49] This similarity suggests that both GAD1 in insects and GAD67 in mammals play critical roles in regulating neuronal inhibition by maintaining basal GABA levels essential for balancing neuronal excitation, which is crucial for preventing neuronal hyperactivity and for modulating behavioral responses to stressful stimuli, including noxious ones.[50]

In our study, the increase in GAD1 expression in bumble bees exposed to noxious stimuli mirrors findings in mammals, where GAD67 supports inhibitory neurotransmission under conditions of stress or pain.[51–55] This increase in GAD1 likely reflects an enhanced GABA production, which helps regulate neuronal activity by diminishing the intensity of nociceptive perception. These findings highlight that, even in insects, GABAergic modulation plays a crucial role in the behavioral response to adverse stimuli, underscoring the conserved nature of GABAergic regulation across species. Consistent with these observations, *Drosophila melanogaster* also uses GAD1 to regulate inhibitory synaptic transmission and modulate nociceptive behavior following injury, contributing to GABA production that mitigates central excitatory responses and maintains inhibition during neuropathic pain or allodynia.[26,56] Following injury, GAD1 expression decreases in the ventral region of the central nervous system, leading to a loss of inhibitory control and the onset of neuropathic allodynia.[26] As for the other genes analyzed here, CaMKII (Ca^{2+}/calmodulin-dependent protein kinase II), which is involved in synaptic plasticity and learning and memory processes,[57–59] did not show significant changes. This finding is consistent with the notion that CaMKII activation is often associated with long-term plastic changes rather than immediate responses to acute stimuli.[57] Its activation typically requires sustained calcium influx, which might not have been sufficiently triggered by the short-term nociceptive stimulus applied in this study. Similarly, the expression of GABAr (GABA receptor, which can be either GABAa or GABAb), responsible for mediating the inhibitory effects of GABA in the central and peripheral nervous systems,[60] did not change significantly. It is possible that GABA receptor expression is only altered under more chronic or prolonged exposure to stimuli. In particular, specific subtypes of GABA receptors could respond differently to nociceptive stimuli, and our analysis might not have captured these nuanced changes. For instance, GABAa receptors are known to mediate fast inhibitory synaptic transmission, while GABAb receptors have slower effects.[61] Differentiating between these subtypes in future studies could provide more detailed insights into receptor-level regulation during nociception. The expression of SYN (synapsin), a synaptic vesicle-associated protein that regulates neurotransmitter release and plays an important role in synaptic plasticity,[62–64] showed a trend toward reduction in the *Experimental* group, although not significant ($p = 0.10$). This reduction could indicate a decrease in synaptic plasticity in response to nociceptive stimulation. Finally, ABAT (4-aminobutyrate aminotransferase), an enzyme involved in GABA catabolism that converts GABA into succinic semialdehyde,[65,66] did not show significant changes in expression, although there appears to be a trend toward an increase in individuals exposed to

Table 2. qPCR primers

Type of gene	Gene	Gene ID	Primer sequence 5' →3'	Product size (bp)	Efficiency (E, %)	Function
target	Abat	100648639	CGCCGACTACGAAAATCAG (For) ATTAACCGGATCAGCGAGAG (Rev)	145	99.0	Involved in GABA catabolism[65,66]
target	Camkii	100648089	CGGCCAGAGATTTTCAGAAG (For) CGAGGTAATGGTGGTTTTCC (Rev)	113	93.3	Involved in synaptic plasticity and learning and memory processes[57–59]
target	GABAr	100645717	GCGGCGTTACCGAAAATCTC (For) CCTTGAACCGCACCTCAGTT (Rev)	267	89.4	Mediates inhibitory effects of GABA in the nervous systems[60]
target	gad1	100649229	TTTGCCAGAGACACAAGCTC (For) TCGTCGAACACTGAAGCAAC (Rev)	164	77.9	Regulates neuronal inhibition by maintaining basal GABA levels.[26,50]
target	Syn	100648875	ATCCGGTTGTGGTGAAACTC (For) CGTGAACGTCGTATTTGGTG (Rev)	155	98.7	Regulates neurotransmitter release and synaptic plasticity[62–64]
Ref.	EF1	100631080	CGTATGCGCATGTCACTTCA (For) GAACCTTTGCCCATTTCCTGG (Rev)	189	102.5	Polypeptide elongation at the ribosome[67]
Ref.	RPS18	100642537	TGCAGCAAGATGTCGCTTGT (For) CGACGACCCGTAGTCTTTGT (Rev)	158	94.7	Participates to the ribosomal functions[68,69]

Primers used for qPCR experiments.

nociceptive stimulation. This trend may balance increased GABA synthesis due to GAD1 upregulation, preventing excessive GABA accumulation and maintaining neurotransmitter equilibrium. It is possible that the lack of significance in ABAT expression is due to the timing of sample collection; a gradual increase in ABAT expression might occur after the initial upregulation of GAD1, suggesting that a delayed sample collection might have captured different outcomes in ABAT expression.

To sum up, our research has allowed us to outline a specific ethogram for the nocifensive behaviors in bumble bees, showing that these insects exhibit behaviors like those of vertebrates when exposed to noxious stimuli. We have also observed how the nocifensive ethogram of bumble bees is altered by a centrally acting GABAergic drug: gabapentin suppresses these responses. Finally, we measured an upregulation of *GAD1* in the brains of a subset of the bumble bees exposed to noxious stimulation and tested behaviorally. Taken together, these molecular findings, combined with the behavioral modifications observed following gabapentin administration, support the hypothesis that GABA plays a key role in modulating nocifensive behaviors in insects. The ability of bees to modulate their behavioral response to a noxious stimulus through GABAergic mechanisms suggests that insects may possess a more complex pain modulation system than previously thought, potentially involving central processes that extend beyond mere peripheral nociception.

These data provide behavioral, pharmacological, and molecular evidence for the presence of analgesia in bumble bees, one of the eight fundamental criteria for demonstrating the presence of pain, and potentially increase the level of confidence in this criterion in *Hymenoptera*, which currently has a medium level of confidence.[19] Specifically, our data suggest that GABA may act as an endogenous neurotransmitter modulating responses to noxious stimuli in these animals. The GABAergic modulation of nocifensive behaviors highlighted by our results supports the idea of the existence of a subjective experience of pain in bumble bees that goes beyond mere nociception and may suggest similarity to the broader definition used in higher vertebrates.

Limitations of the study

Only worker bumble bees were used in our study, as the experimental protocol was designed around the foraging behavior of bees. Consequently, only females were included in our experiments and this may limit the generalizability of the results, as they cannot be directly extended to male bumble bees, whose behavioral and neurobiological responses may differ.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Elisa Pasquini, (elisa.pasquini@unitn.it).

Materials availability

This study did not generate new materials.

Data and code availability

- Raw data have been deposited at Mendeley Data and are publicly available as of the date of publication. DOIs are listed in the key resources table.
- All original code has been deposited at Mendeley Data and is publicly available as of the date of publication. DOIs are listed in the key resources table.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization, E.P., D.B., and E.F.; methodology, E.P., D.B., A.M., and E.F.; investigation, E.P., J.B., and V.D.R.; visualization, E.P.; data analysis, E.P. and E.F.; supervision, E.F.; funding acquisition, E.F.; writing—original draft, E.P.; writing—review and editing, E.P., D.B., A.M., and E.F.

DECLARATION OF INTERESTS

Authors declare that they have no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Bumble bee brains (*Bombus terrestris*)	Bioplanet, Cesena, Italy	
Chemicals, peptides, and recombinant proteins		
Gabapentin	Sigma-Aldrich	CASID#60142-96-3
Critical commercial assays		
Arcturus Picopure RNA Isolation Kit	ThermoFisher Scientific	Cat#KIT0204
Deposited data		
Raw data	Mendeley Data	https://doi.org/10.17632/4ghnf7k6nc.1
Code	Mendeley Data	https://doi.org/10.17632/4ghnf7k6nc.1
Experimental models: Organisms/strains		
Bumble bee (*Bombus terrestris*)	Bioplanet, Cesena, Italy	
Oligonucleotides		
Abat	Sigma-Aldrich	GeneID#100648639
CaMKII	Sigma-Aldrich	GeneID#100648089
GABAr	Sigma-Aldrich	GeneID#100645717
gad1	Sigma-Aldrich	GeneID#100649229
Syn	Sigma-Aldrich	GeneID#100648875
EF1	Sigma-Aldrich	GeneID#100631080
RPS18	Sigma-Aldrich	GeneID#100642537
Software and algorithms		
BORIS software (version 7.9.15)	Friard & Gamba, 2016[70]	
Bio-Rad CFX Manager	Bio-Rad	
R software	https://www.r-project.org/	R version 4.3.0

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

The experiments, conducted from June 2023 to July 2024, involved female worker bumble bees, *Bombus terrestris*, from nine commercially farmed colonies (Bioplanet, Cesena, Italy). During all the experiments, the bees were maintained in their colony within a controlled lab environment at the Animal Cognition and Neuroscience laboratories of CIMeC, University of Trento, with a constant temperature of 23 ± 2°C. In addition to the sucrose solution given to some specific workers during the tests, 10 mL of 50% (w/v) sucrose solution was placed in the nest every evening and 7 g of pollen was also provided every two days.

METHOD DETAILS

Ethics

There are currently no regulation governing the welfare of insects in research. Nevertheless, our experimental approach was guided by the principles of the 3Rs (Refinement, Reduction, Replacement) and the Animal Sentience Precautionary Principle (ASPP).[71,72] Despite the need of noxious stimuli in pain studies, we opted for a temperature of 50°C which has no lasting impact on the well-being of the bees, based on previous literature on bumble bees.[20] We tested a number of bees that was sufficient to allow us generalization and statistical significance of results. For brain data extraction (RT-qPCR), we minimized bumble bee sacrifices while ensuring that the data collected would provide statistically significant results, using sample sizes typical for qPCR experiments to ensure validity and reliability.

Experimental set-up

Each colony was placed into a custom-made set-up consisting of a ventilated wooden nest box (46 × 36 × 32 cm; l × w × h) connected to the rest of the apparatus (Figures 5 and S1). The bees were free to emerge from the colony into a Plexiglass chamber (14.5 × 14.5 × 14.5 cm). This chamber was connected to a Plexiglass foraging area (20 × 20 × 25 cm) through a corridor in which five vertical sliding doors allowed the experimenter to selectively release one at the time the tested bees into the foraging area. To stabilize flight, the foraging area was internally lined with a random red (perceived as gray to the bees) and white checkerboard

pattern of 5 mm squares. The corridor consisted of two Plexiglass tunnels, one connected to the Plexiglass chamber (21 × 5 × 5 cm) and the other to the foraging area (15 × 5 × 5 cm). These two Plexiglass tunnels were connected by a heat conducting tunnel (12 × 2 × 2 cm) made of aluminum and representing the noxious stimulus the bees received during the tests (hereafter referred to as *nociceptive tunnel*). The nociceptive tunnel was placed in a wooden frame (12 × 5.5 × 3.5 cm) and connected to a modulated heat generator via a custom-made system composed of two conductive pads (5 × 0.5 × 3 cm) and a feedback probe (Figures 5 and S2).

The experimental set-up was designed to observe the behavior of the bees in response to the nociceptive tunnel during their return to the colony after foraging. For this purpose, two video cameras (The Imaging Source DMK 27BUR0135) with a TAMRON lens (5.0–50 mm F/1.4) were hung 40 cm above two observation areas (9 × 5 × 5 cm), i.e., the two plexiglass tunnels adjacent to the nociceptive tunnel. We refer to the first tunnel as latency observation area and to the second tunnel as behavioral observation area.

Experimental procedure

After being placed into the set-up, each colony had three days to familiarize with the apparatus. During this period, all sliding doors were removed to allow the bees to freely explore the apparatus and access the foraging area, where they could feed from a blue artificial flower (5 × 4.5 cm; l × w) with an Eppendorf lid glued on it (1 × 0.5 cm; d × h) containing a 50% (w/v) sugar solution. At this stage, the best-performing foragers of approximately the same size (~0.40 g) were identified and marked with different water colored Uniposca to be used in the test. The bees never experienced heat during this familiarization phase.

Each marked bee was selected one at the time and, after feeding from the foraging area, was subjected to the test during its return to the colony. The colony was used as an attractive stimulus, while the nociceptive tunnel represented the obstacle to re-join the colony after foraging. The test consisted of passing through the nociceptive tunnel, either switched-off or heated up to a constant temperature of 50°C (the temperature was monitored using a Bosch infrared thermometer) after which the bee was constrained in the observation area and its behaviors were video-recorded for 5 min. Following this observation period, the bee was allowed to go back to the colony. We identified the behaviors performed by each bee during the 5 min spent in the observation area. We also recorded the *latency*, defined as the time it takes for each individual bee to enter the tunnel. Latency refers specifically to the time from the moment the bee enters the latency observation area, until the bee enters the nociceptive tunnel. Thus, latency does not include the time taken to cross the tunnel itself. We allowed each bee 5 min to enter the nociceptive tunnel; bees that did not enter the tunnel within the initial 5 min were attracted into the observation area using UV light (Darkbeam, A300-365nm).

Exp 1 - Identification and quantification of nocifensive behaviors

Thirty-five individual foragers were first tested with the nociceptive tunnel switched-off (control phase - unheated tunnel condition) to characterize the spontaneous behavior as control. The same bees were then tested in a second phase with the nociceptive tunnel heated up to 50°C (experimental phase - heated tunnel condition) to evaluate the nocifensive behaviors induced by the nociceptive stimulation. After each trial, the experimental set-up was cleaned with a 30% (w/v) ethanol solution and the artificial flower was refilled.

The observation of the behaviors of the bumble bees in the 5-min after passing through the tunnel allowed us to identify the spontaneous behaviors of the bees in the control phase and the nocifensive behaviors of the same bees in the experimental phase. The ethogram shown in Table 1 details the eight identified behaviors. Video samples of each performed behavior are in the supplemental information.

Exp 2 – Effect of drug manipulation on nocifensive behaviors

Preparation of drug-containing sucrose solutions

Gabapentin (Sigma-Aldrich) was dissolved in 50% sucrose solution (w/v) to make a final concentration of 0.24 mg/mL. The solution was administered orally to individual bees in volumes of 5 μL using a Gilson P20 pipette. The administered dose of gabapentin (0.12 mg per bee) was determined based on those used in rats, corresponding to 300 mg/kg.[35]

Testing with drug administration

Experiment 2 involved 138 bees distributed as follows: 70 individuals were treated with 5 μL of sucrose solution + Gabapentin (35 control and 35 experimental bees), and 68 individuals were tested with the same amount of sucrose solution but without the drug (34 control and 34 experimental bees). To administer the solutions, when the bees reached the foraging area, they were captured using a marking cage. At this stage, the sucrose solution containing the drug (or without the drug) was administered orally to the bee inside the cage to ensure complete ingestion. Following this step, each bee was reintroduced into the foraging area and confined there for 1 h to allow the drug to be absorbed and to achieve maximum efficacy.[35] At the end of this period, all bees had to pass through the nociceptive tunnel, which was either switched off (control bees) or heated at a constant temperature of 50°C (experimental bees). After the observation period of 5 min, the bees treated with Gabapentin were removed from the experimental apparatus and placed in a separate cage. In this set-up, their survival was monitored during the hours immediately following the test and daily for up to 72 h post-treatment. The treated bees, however, remained confined in this new setup until their natural death, enabling prolonged observation of survival for up to 2–3 weeks. This parameter was monitored by observation, but was not systematically recorded.

Behavioral analysis

The behavioral analysis focused on measuring both the latency (s) and the duration of each behavior (s) over the 5-min period using the behavior analysis software BORIS (version 7.9.15; Friard & Gamba, 2016). Latency measurement could be objectively determined, but quantifying behaviors could involve subjective interpretation. For this reason, to ensure a more accurate quantification of the time spent performing each behavior, 70% of video clips was analyzed by two independent coders blind to the experimental conditions. In total, three experimenters were involved in this process. The inter-rater reliability between the two coders was assessed with the Spearman-rho ranked-order correlation coefficient.[73] Although the raw data scored by the different experimenters was similar in all cases with "strong" and "very strong" positive ρ (Exp1 $\rho = 0.77$; Exp 2: *Gabapentin* $\rho = 0.89$), we considered the mean value between the time recorded by the two experimenters. Some behaviors performed simultaneously by the bees were quantified together using BORIS, i.e., walking + moving wings, and resting + ventilating, and then separated for statistical analysis.

Exp 3 – Quantification of GABA genes expression

Samples collection, brain dissection and total RNA extraction

The bees used for molecular analyses were randomly collected from the control and the experimental groups of Exp 2 treated only with sucrose solution. Immediately after the 5-min behavioral observation period, six bees from the control group (tunnel off) and six bees from the experimental group (tunnel on) were first anesthetized with CO_2 and then sacrificed on dry ice. Samples were stored at - 80°C prior to dissection. The frozen bee brains were dissected on dry ice under a microscope,[74] and the samples were kept at - 80°C until RNA extraction. RNA was extracted and purified using the Arcturus Picopure RNA Isolation Kit (ThermoFisher Scientific).[75] The total RNA purity (A260/A280 and A260/230 values) and concentration were measured using a Nanodrop spectrophotometer (Nanodrop OneC; ThermoFisher Scientific). For reverse transcription, 15 μL of solution containing 10.5 μL of RNA, 3 μL of buffer, and 1.5 μL of enzyme mix was used with the SuperScript VILO cDNA Synthesis Kit (Invitrogen, ThermoFisher Scientific).

Target genes selection process

The selection of the five GABA-related genes analyzed in this study was based on prior research conducted on *Apis mellifera*, where these same markers were used to investigate the expression of GABA-related genes in the context of learning performance.[28] The primers for the RT-qPCR were designed using the BLAST Primer Design Tool and the gene sequences on which the primers were to match were retrieved from the NCBI gene database using the reference gene of *Bombus terrestris*. For each of the five selected genes, orthologs in *Bombus terrestris* were identified to ensure accurate targeting of species-specific sequences. These genes were selected because they represent key stages in the GABAergic pathway, allowing us to capture a comprehensive view of GABA metabolism - from synthesis to release and consumption. Specifically, the selected markers include: GAD1 (Glutamate decarboxylase) for GABA synthesis, ABAT (4-Aminobutyrate aminotransferase) for catabolism, GABAr (GABA receptor) for receptor-mediated signaling, CaMKII (Ca2+/calmodulin-dependent protein kinase II) for synaptic plasticity, and SYN (Synapsin) for synaptic plasticity and neurotransmitter release. Two reference genes (Ef1α and RPS18) were used for normalization as previously reported for other *Hymenoptera*.[76,77] The complete list of primers was reported in Table 2.

Quantitative polymerase chain reaction (RT-qPCR)

Each sample was run in triplicate using the PowerUp SYBR Green Master Mix (2X). Real-time quantitative PCR was performed in 96-well PCR plates (Bio-Rad) sealed with Microseal 'B' PCR Plate Sealing Film (Bio-Rad) and run on a CFX96 Real-Time System (Bio-Rad). The data were then analyzed using Bio-Rad CFX Manager software. Gene expression quantification was carried out using the ΔCq method.[78] The data were normalized against the expression of the two reference genes (ΔCq), and the relative expression of each target gene was calculated accordingly.

QUANTIFICATION AND STATISTICAL ANALYSIS

The statistical analysis of the data was carried out using the R software (version 4.3.0) with the RStudio interface (version 2023.03.1). We checked the distribution of our data by using Shapiro-Wilk test. Since the data were not normally distributed, we used a paired Wilcoxon signed rank test (Exp 1) and Kruskal-Wallis test followed by Dunn's post-hoc test with Bonferroni correction (Exp 2) to assess the differences in terms of latency. To test for differences among behaviors, the distribution of the data was first visually assessed (hist) and then, to improve normality and to reduce heteroscedasticity, we used the Box-Cox transformation for the analysis.

In Exp 1, to analyze the effects of *Type* and *Behavior* on the transformed dependent variable *Duration*, we initially used a linear mixed-effects model with *Subject* as a random effect to account for individual variability. However, the variance associated with *Subject* was consistently zero, indicating negligible between-subject variability and suggesting that the observed variability is primarily explained by the fixed effects included in the model (*Type* and *Behavior*), rather than intrinsic characteristics of the subjects. Therefore, we proceeded with a simpler linear model (LM), which included *Type*, *Behavior*, and an interaction term, as well as a *Dummy* variable to account for the presence of zero values in the data. The *Dummy* variable represents the absence of the observed behavior, and consequently, when this absence occurs, the *Duration* is zero.

In Exp 2, we extended the model to include an additional independent variable, *Drug*, alongside *Type* and *Behavior*. The model for Exp 2 also initially included *Subject* as a random effect, but due to zero variance, the random effect was removed, and an LM was used instead. The final model included *Type*, *Behavior*, *Drug*, their interaction terms, and a *Dummy* variable for zero values. As the behaviors were not mutually exclusive, we tested each of the behaviors independently, to better understand the impact of the

experimental conditions on each specific behavior. Therefore, given that the data did not meet parametric assumptions we used Wilcoxon tests (paired for Exp 1 and non-paired for Exp 2) to assess the differences for each specific behavior separately.

For graphical purposes, both latency and behavioral data were transformed using the sqrt method to improve data visualisation, and the dependent variable *Duration* was renamed on the y axis as "Latency (sec)" for latency and "Behavior Duration (sec)" for behavioral data.

In Exp 3, we checked the distribution of the data for each gene using the Shapiro-Wilk test. Since the data deviated from normality in some cases, a Box-Cox transformation was applied. In addition to approximating normality, the transformation stabilised the variance, allowing the use of parametric tests. The gene expression of each gene was compared between the control and experimental group using two-sample t-tests.

In all statistical analyses, a p-value <0.05 was considered significant.