Cofactor-enabled functional expression of fruit fly, honeybee, and bumblebee nicotinic receptors reveals picomolar neonicotinoid actions

Makoto Ibara,1,2,3* Shogo Furutani,1,2 Shota Shimada,3 Kunihiro Niki,4 Yuma Komori,4 Masaki Kamiya,5 Wataru Koizumi,5 Leo Magara,5 Mai Hikida,5 Akira Noguchi,5 Daiki Okuhara,5 Yuto Yoshinari,5 Shu Kondo,5 Hiromu Tanimoto6,7 Ryusuke Niwa8,9, David B. Sattelle1,9, and Kazuhiro Matsuda1,9,2

*Department of Applied Biological Chemistry, Faculty of Agriculture, Kindai University, Nara 631-8505, Japan; 1Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8577, Japan; 2Genetic Strains Research Center, National Institute of Genetics, Mishima, Shizuoka 411-8540, Japan; 3Graduate School of Life Sciences, Tohoku University, Sendai, Miyagi 980-8577, Japan; 4Life Science Center for Survival Dynamics, Tsukuba Advanced Research Alliance, University of Tsukuba, Tsukuba, Ibaraki 305-8577, Japan; 5Centre for Respiratory Biology, University College London Respiratory, University College London, London WC1E 6JF, United Kingdom; and 6Agricultural Technology and Innovation Research Institute, Kindai University, Nara 631-8505, Japan

Edited by John G. Hildebrand, University of Arizona, Tucson, AZ, and approved May 18, 2020 (received for review February 26, 2020)

The difficulty of achieving robust functional expression of insect nicotinic acetylcholine receptors (nAChRs) has hampered our understanding of these important molecular targets of globally deployed neonicotinoid insecticides at a time when concerns have grown regarding the toxicity of this chemotype to insect pollinators. We show that thioredoxin-related transmembrane protein 3 (TMX3) is essential to enable robust expression in Xenopus laevis oocytes of honeybee (Apis mellifera) and bumblebee (Bombus terrestris) as well as fruit fly (Drosophila melanogaster) nAChR heteromers targeted by neonicotinoids and not hitherto robustly expressed. This has enabled the characterization of picomolar target site actions of neonicotinoids, findings important in understanding their toxicity.

neonicotinoids | nicotinic acetylcholine receptors | fruit fly | honeybee | bumblebee

Neonicotinoid insecticides display selective actions on insect nicotinic acetylcholine receptors (insect nAChRs) and show high plant systemic activity that enables seed treatment (1–5). Hence, for the past two decades, they have been widely used for plant protection and animal health care (3). However, adverse actions of neonicotinoids on bee behavior, colony size, and queen production have been demonstrated (6–13). Their intensive use also correlates with reduced numbers of insectivorous birds (14). Hence, on April 27, 2018, the European Union (EU) placed a ban on outdoor use of the most commonly used neonicotinoids (imidacloprid, thiamethoxam, and clothianidin). There were also calls for wider international restrictions of neonicotinoid use (15). It is therefore urgent to understand the mechanism of neonicotinoid actions and toxicity.

A major barrier to achieving this goal has been the challenge of obtaining robust, heterologous functional expression of cloned insect nAChR subunits using well-established expression vehicles such as Xenopus laevis oocytes or Drosophila melanogaster S2 cells. It is difficult to reliably achieve heterologous expression of insect nAChRs, but insect α-subunits form robust nAChRs when coexpressed with certain vertebrate non-α-subunits (16). These insect/vertebrate hybrid nAChRs have been employed to study the mode and diversity of neonicotinoid actions (5, 17, 18). However, some features of insect native nAChR interactions with neonicotinoids are not easily studied using such hybrid nAChRs. For example, studies on honeybee (Apis mellifera) cultured antennal lobe neurons (19, 20) and Kenyon cells (21) as well as on cockroach (Periplaneta americana) (22) and fruit fly (D. melanogaster) cholinergic neurons (23) showed that imidacloprid acts as a partial agonist on nAChRs. Imidacloprid blocked the desensitizing component of native nAChRs on cockroach neurons (24), acting selectively on one receptor subtype, whereas clothianidin activates two distinct receptor subtypes (25). In all cases, the subunit composition and stoichiometry of native insect nAChRs is unknown. There is an urgent need to quantify the actions of neonicotinoids, notably those restricted for crop protection use in the EU, on insect nAChRs of known subunit composition. We report that insect nAChRs of the fruit fly (D. melanogaster), the western honeybee (A. mellifera), and the buff-tailed bumblebee (Bombus terrestris) can be expressed robustly in X. laevis oocytes with the aid of cofactors, notably TMX3 (26). We show that heteromeric honeybee and bumblebee nAChRs are sensitive to picomolar imidacloprid, thiacloprid, and clothianidin, counseling caution for continued neonicotinoid use in the field.

Significance

Neonicotinoids acting on insect nicotinic acetylcholine receptors (nAChRs) are deployed for crop protection, but growing evidence for adverse effects on insect pollinators has led to restricted use of some neonicotinoids in the EU. It is therefore vital to understand the target site actions of neonicotinoids in pollinators, but to date the difficulties of heterologous expression of insect nAChRs have hampered progress. We have found that a thioredoxin (TMX3) enables robust functional expression of honeybee, bumblebee, and fruit fly nAChRs in Xenopus laevis oocytes. With this advance, we show that expressed bee nAChRs are more neonicotinoid-sensitive than those of fruit fly, and clothianidin can modulate both honeybee and bumblebee nAChRs at a concentration below that commonly observed in agricultural fields.

Author contributions: M.I., R.N., D.B.S., and K.M. designed research; M.I., S.F., S. Shigetou, S. Shimada, K.N., Y.K., M.K., W.K., L.M., A.N., D.O., Y.Y., S.K., H.T., R.N., and K.M. performed research; M.I. and K.M. analyzed data; and M.I., R.N., D.B.S., and K.M. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

This open access article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

Data deposition: The sequence data reported in this paper have been deposited to DNA Data Bank of Japan (accession nos. BCD56239, BCD56240, and BCD56241).

1M.I. and S.F. contributed equally to this work.

2To whom correspondence may be addressed. Email: kmatsuda@nara.kindai.ac.jp.

This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2003667117/-/DCSupplemental.
Results and Discussion

Functional Expression of Insect nAChRs. Our initial attempts at robust functional expression of insect nAChRs focused on the D\(\alpha_1\), D\(\alpha_2\), D\(\beta_1\), and D\(\beta_2\) subunits of D. melanogaster since biochemical studies point toward their coassembly (27, 28). To confirm their colocalization, we explored the expression of these nAChR subunit genes using a viral T2A peptide-mediated GAL4 transgenic knockin (29, 30) to drive the expression of a membrane-tethered GFP reporter gene. We employed the previously identified octopaminergic neurons innervating the testis ejaculatory duct (Fig. 1A) (31), since their neurites are easily identified by visualizing with membrane-tethered GFP and anti-tyrosine decarboxylase 2 (Tdc2) antibody immunostaining. We found that a GFP signal reflecting the expression of the D\(\alpha_1\) gene was detected in Tdc2-positive neurites (Fig. 1B). Using the same method, we showed that D\(\alpha_2\), D\(\beta_1\), and D\(\beta_2\) genes were also expressed in the same neurons (Fig. 1C–E). Considering that GFP signals driven by each of four nAChR subunit genes overlapped almost perfectly with the Tdc2 signal in these neurons, these results suggest that the four D. melanogaster subunits are likely to coexist in single neurons targeting the ejaculatory duct.

We next explored the expression of D\(\alpha_1\) and D\(\beta_1\) subunits selected as a minimal heteromeric subunit combination in X. laevis oocytes. Despite evidence of their colocalization, however, we found no electrophysiological evidence of functional expression, findings resembling those previously reported in experiments using D. melanogaster S2 and human embryonic kidney (HEK293) cells as expression vehicles (32). In the nematode Caenorhabditis elegans, RIC-3 and UNC-50 promote nAChR maturation (33) and nAChR trafficking (34), respectively. We therefore examined whether a simultaneous introduction of these regulators influence fruit fly nAChR expression in X. laevis oocytes. However, no successful expression of the D\(\alpha_1/D\beta_1\) nAChR was observed, not even when we cojected this subunit pairing, together with cRNAs encoding the nAChR subunits, cRNAs encoding the D. melanogaster orthologs of RIC-3 (DmRIC-3, CG30926) and UNC-50 (DmUNC-50, CG9773) (SI Appendix, Fig. S1). We postulated that a missing component for robust insect nAChR expression could be a thioredoxin, possibly underlying the disulfide bond formation of the Cys-loop superfamily proteins critical for subunit assembly and the coupling of ligand binding to channel gating (35, 36). We therefore coexpressed thioredoxin-related transmembrane protein 3 (DmTMX3)
Table 1. Agonist actions of acetycholine and neonicotinoids on fruit fly, honeybee, and bumblebee nAChRs

| nAChRs          | Acetylcholine | Imidacloprid | Thiacloprid | Clothianidin |
|-----------------|---------------|--------------|-------------|--------------|
| pEC50           | pEC50         | lmax         | pEC50       | lmax         |
| pEC50           | pEC50         | lmax         | pEC50       | lmax         |
| Fruit fly (D. melanogaster) |               |               |             |               |
| Dx1/D1β1        | 5.12 ± 0.02** | 6.76 ± 0.23ab | 0.112 ± 0.009 | 7.64 ± 0.18ab | 0.065 ± 0.005 |
| Dx1/D1β(R81T)   | 5.48 ± 0.04   | 6.06 ± 0.12   | 0.032 ± 0.002 | 7.01 ± 0.09   | 0.015 ± 0.001 |
| Dx1/D2β/D1β    | 4.29 ± 0.04ab | 6.39 ± 0.18   | 0.046 ± 0.004ab | 6.63 ± 0.17  | 0.020 ± 0.002bc |
| Dx1/D2β/D1β(R81T) | 6.48 ± 0.04 | 5.76 ± 0.15   | 0.023 ± 0.002 | 6.44 ± 0.18 | 0.017 ± 0.002 |
| Dx1/D2β/D1β    | 4.82 ± 0.02   | 6.97 ± 0.21ab | 0.244 ± 0.022 | 7.73 ± 0.19ab | 0.056 ± 0.005ab |
| Dx1/D2β(D1β/R81T) | 5.33 ± 0.03 | 5.65 ± 0.25b  | 0.025 ± 0.003 | 7.29 ± 0.09 | 0.0057 ± 0.003 |
| Dx1/D2β/D1β(D1β/R81T) | 5.22 ± 0.07 | 6.92 ± 0.12ab | 0.592 ± 0.030 | 7.15 ± 0.08bc | 0.454 ± 0.019 |
| Dx1/D2β(D1β/R81T) | 4.95 ± 0.04 | 5.69 ± 0.18  | 0.811 ± 0.006 | 7.07 ± 0.12 | 0.014 ± 0.001 |
| Honeybee (A. mellifera) |               |               |             |               |
| Amu1(Amu2/Amu1) | 5.94 ± 0.04   | 7.63 ± 0.13ab | 0.075 ± 0.004 | 7.94 ± 0.19ab | 0.058 ± 0.006ab |
| Amu1(Amu2/Amu1) | 5.72 ± 0.03   | 7.48 ± 0.16   | 0.070 ± 0.005 | 8.16 ± 0.13b  | 0.037 ± 0.003bc |
| Bumblebee (B. terrestris) |               |               |             |               |
| Btu1(Btu8/Btu1) | 5.80 ± 0.03ab | 7.60 ± 0.26ab | 0.107 ± 0.010 | 7.29 ± 0.26abc | 0.094 ± 0.008ab |
| Btu1(Btu8/Btu1) | 5.66 ± 0.03  | 7.40 ± 0.20ab | 0.085 ± 0.007a | 7.14 ± 0.38ac | 0.092 ± 0.011 |

Data are represented as mean ± SEM (n = 5).

*Indicates that data for the R81T mutant differ from that for the corresponding wild-type nAChR in D. melanogaster (two-way ANOVA, Bonferroni test, P < 0.05).

**Indicates that data for the R81T mutant differ from that for the corresponding wild-type nAChR in D. melanogaster (two-way ANOVA, Tukey test, P < 0.05).

†Indicates that data for the R81T mutant differ from that for the corresponding wild-type nAChR in D. melanogaster (one-way ANOVA, Tukey test, P < 0.05).
that, in these expressed insect nAChRs, the Dβ1 subunit is a key player in neonicotinoid interactions.

In native insect neurons, imidacloprid attenuates the desensitizing component more profoundly than the nondesensitizing component of ACh responses at low concentrations (24). Hence, we investigated the effects of coapplication with the neurotransmitter of imidacloprid, thiacloprid, and clothianidin at concentrations <10 nM, below the threshold for agonist actions, on the ACh-induced response of the D. melanogaster recombinant nAChRs (Fig. 2 E–H). When imidacloprid was applied at
1 nM for 1 min followed immediately by coapplication with ACh for 2 min, it attenuated the fast desensitizing component of the ACh response of the D\(\alpha 1/D\beta 1\), D\(\alpha 1/D\alpha 2/D\beta 1/D\beta 2\) nAChRs, while scarcely influencing a nondesensitizing component (SI Appendix, Fig. S5). Thiacloprid reduced the ACh response of the D\(\alpha 1/D\beta 1\) nAChR even at 100 pM (Fig. 2E) and was more potent than imidacloprid and clothianidin in its antagonist action on the D\(\alpha 1/D\beta 1/D\beta 2\) nAChR response (Fig. 2G; \(P < 0.05\) [one-way ANOVA, Bonferroni test]). Clothianidin tested at 1 nM blocked the ACh response of the D\(\alpha 1/D\beta 1\) and D\(\alpha 1/D\alpha 2/D\beta 1\) nAChRs (Fig. 1E and F; \(P < 0.05\) [one-way ANOVA, Bonferroni test]), while being ineffective on the D\(\alpha 1/D\beta 1/D\beta 2\) and D\(\alpha 1/D\alpha 2/D\beta 1/D\beta 2\) nAChRs (Fig. 2G and H). The R81T mutation in the D\(\beta 1\) subunit attenuated the antagonist actions of the neonicotinoids on all of the \(D.\) \(mellonogaster\) nAChRs (Fig. 2E–H), confirming the role of the D\(\beta 1\) subunit in determining the antagonist activity of neonicotinoids as well as their agonist activity.

**Target Site Actions of Neonicotinoids in Honeybees and Bumblebees.**

Based on the findings in the fruit fly, we attempted to address neonicotinoid actions on heteromeric nAChRs in two pollinator species, the western honeybee (\(A.\) \(mellifera\)) and the buff-tailed bumblebee (\(B.\) \(terrestris\)). We first examined the capacity of the three auxiliary proteins RIC-3, UNC-50, and TMX3 to express the nAChRs in \(X.\) \(laevis\) oocytes. In this experiment, we employed the \(A.\) \(mellifera\) \(\alpha 8\) (Am\(\alpha 8\)) and B. \(terrestris\) \(\alpha 8\) (Bt\(\alpha 8\)) as the \(\alpha 8\) subunits in both species show the highest amino acid sequence similarity to the fruit fly \(D.\) \(\beta 2\) subunit (Fig. 3A). Coexpressing together Am\(\alpha 1\), Am\(\alpha 8\), and Am\(\beta 1\) subunits or Am\(\alpha 1\), Am\(\alpha 2\), Am\(\alpha 8\), and Am\(\beta 1\) subunits with AmRIC-3, AmUNC-50, and AmTMX3 resulted in the first robust expression of honeybee

**Fig. 3.** Agonist actions of neonicotinoids on honeybee and bumblebee nAChRs. (A) Relationships of \(D.\) \(mellonogaster\), \(A.\) \(mellifera\), and \(B.\) \(terrestris\) nAChR subunit proteins. DmRDL: \(D.\) \(mellonogaster\) GABA\(_A\) receptor subunit RDL. Bootstrap values are shown at each node. (B and C) ACh-induced responses of the honeybee (B) and bumblebee (C) nAChRs. The boxes represent median and 25th to 75th percentiles of ACh response amplitudes with minimum and maximum shown as whiskers (honeybee, \(n = 20\); bumblebee, \(n = 10\)). (D and E) Concentration–response relationships for neonicotinoids on the honeybee (D) and bumblebee (E) nAChRs. Each plot represents mean ± SEM (\(n = 5\)).
nAChRs corresponding to the fruit fly D. melanogaster D1 and D2, and D. rerio D1/D2 α/β nAChRs, respectively, (Fig. 3B). Similarly, robust bumblebee Btx1/Btx8/βIβ1 and Btx1/Btx2/Btx8/βIβ1 nAChRs were formed in X. laevis oocytes in the presence the three equivalent bumblebee cofactors (BtRIC-3, BtUNC-50, BtTMX3) (Fig. 3C).

We evaluated agonist activity of the three neonicotinoids for the A. mellifera and B. terrestris nAChRs. Imidacloprid was a partial agonist as in native insect neurons (19–23) with similar affinity for the honeybee and the bumblebee nAChRs, while thiacloprid acted as a partial agonist with higher affinity for the honeybee Amα1/Amα2/Amβ1 nAChRs compared to the bumblebee Btx1/Btx8/βIβ1 and Btx1/Btx2/Btx8/βIβ1 nAChRs (Fig. 3 D and E and Table 1; P < 0.05 [one-way ANOVA, Tukey test]). Of the commercial neonicotinoids, clothianidin is most widely used for crop protection and has been documented as a factor in the decline of wild bees, honeybees, and bumblebees (6, 10).

Interestingly, clothianidin showed higher agonist affinity not only for the honeybee Amα1/Amα2/Amβ1 and Amα1/Amα2/Amβ1 nAChRs but also for the bumblebee Btx1/Btx8/βIβ1 and Btx1/Btx2/Btx8/βIβ1 nAChRs than most of the fruit fly nAChRs (Fig. 2 A–D vs. Fig. 3 D and E and Table 1; P < 0.05 [one-way ANOVA, Tukey test]). Furthermore, clothianidin showed comparable affinity to thiacloprid and the highest efficacy among the neonicotinoids tested for the honeybee and bumblebee nAChRs (Fig. 3 D and E and Table 1), suggesting that both insect pollinator species possess nAChRs with features favorable for binding this insecticide.

It has been shown that imidacloprid acts as a partial agonist on nAChRs expressed in honeybee neurons (19–21) and that imidacloprid and clothianidin affect the excitability of honeybee (A. mellifera) Kenyon cells at concentrations as low as 10 nM; imidacloprid reduces the peak amplitude of the ACh response of Kenyon cells with an IC50 of 295 nM (38). However, the threshold concentration for neonicotinoid modulation of honeybee and bumblebee nAChRs is not known. From an eco-toxicological perspective, evaluating the target site actions of neonicotinoids at picomolar concentrations is critical as it offers insights into sublethal effects on honeybees and bumblebees at field-relevant concentrations. We therefore examined the effects of imidacloprid, thiacloprid, and clothianidin on the ACh-induced responses of the honeybee and bumblebee nAChRs at picomolar concentrations at which they did not show agonist actions (Fig. 4 and S1 Appendix, Fig. S6). Here, we show that imidacloprid and thiacloprid suppress the peak amplitude of the ACh response of Amα1/Amα2/Amβ1 and Amα1/Amα2/Amβ1 nAChRs of honeybees as well as Btx1/Btx8/βIβ1 and Btx1/Btx2/Btx8/βIβ1 nAChRs of bumblebees at 100 pM and thiacloprid affected not only the honeybee Amα1/Amα2/Amβ1 and Amα1/Amα2/Amβ1 nAChRs (Fig. 4A), but also bumblebee Btx1/Btx8/βIβ1 nAChR even at 10 pM (Fig. 4B; P < 0.05 [one-way ANOVA, Bonferroni test]). Thiacloprid is metabolized faster than imidacloprid in B. terrestris and B. terrestris α/β nAChRs (Fig. 4A, B; P < 0.05 [one-way ANOVA, Bonferroni test]), suggesting a cancer risk to these insect pollinator species.

In conclusion, we have succeeded in robust, functional expression in X. laevis oocytes of honeybee (A. mellifera), bumblebee (B. terrestris), and fruit fly (D. melanogaster) nAChRs using TMX3 as a key cofactor. Our data showing that neonicotinoids have a particularly high impact on the A. mellifera and B. terrestris nAChRs tested are of importance in considering the future of plant protection using neonicotinoids. Of the neonicotinoids restricted in the EU, clothianidin modulates the honeybee and bumblebee nAChRs containing the α8 subunit even at 10 pM, a concentration much lower than that to which bees are exposed in the field. As the Btx8 subunit-containing nAChRs underpin bee olfactory retrieval behavior (41), chronic exposure to sublethal doses of neonicotinoids could lead to abnormalities in synaptic timing and thereby alter this important bee behavior. Our discovery of these sublethal, picomolar actions of neonicotinoids on their targets shows precisely how cholinergic signaling by the insect neurotransmitter ACh is modified. Parasites, viral pathogens, climate change, habitat loss, and alien species can be co-controllers with neonicotinoids to the decline of bees (42, 43). It is therefore necessary to regulate neonicotinoids carefully and to consider all of the stressors in the environment.

We acknowledge that for nAChRs expressed in X. laevis oocytes, the lipid environment differs from that of insect nerve, as do pretranslation and posttranslation modifications, and codon bias differences may also exist. Also, we have only studied neonicotinoid actions on some insect nAChRs. Therefore, more work is needed on how α- and non-α-subunits are assembled to form nAChRs in particular neurons and to what extent their expression is modulated by developmental and environmental factors. Also, it is necessary to evaluate in more detail the range of actions not only of neonicotinoids, but also of new insecticides targeting insect nAChRs when expressed not only in X. laevis oocytes, but also in other expression vehicles. Nevertheless, our discovery of the importance of cofactor TMX3 in enabling robust insect nAChR expression offers a route to functional studies on nAChRs, not only those of other beneficial insects, insect pests, and disease vectors, but also those of non-target terrestrial and aquatic invertebrates, which have so far proved highly challenging and in many cases elusive.

Materials and Methods

Chemicals. Imidacloprid, thiacloprid, and clothianidin were purchased from FUJIFILM Wako Pure Chemical. ACh and atropine were purchased from MilliporeSigma.

DNA Cloning. In addition to DNA encoding D. melanogaster D1 (NP_524841), Dn2 (NP_524482), Dji1 (NP_523927), Dji2 (NP_524483), DmRIC-3 (CAP16647), DmUNC-50 (NP_649813), and DmTMX3 (NP.648847), those encoding A. mellifera Amα1 (NP.026298411), Amα2 (NP.00101625), Amβ1 (NP.0011575), Amα1 (NP.00103028), Amα3 (BCD56240), Amα5–Amα6 (AE070276), and Amβ1 (BCD56241) and those encoding B. terrastris BtRIC-3 (XP.00339561), BtUNC-50 (XP.012166790), Btx8 (XP.012163744), Btx1 (XP.003393394), BtRIC-3 (BCD56239), BtUNC-50 (XP.012167154), and BtTMX3 (XP.004303488) were cloned into pCDNA3.1 (+) vector (Thermo Fisher Scientific).

Functional Expression of nAChRs and Electrophysiology. Female X. laevis were anesthetized and oocytes were obtained according to the UK Animals (Scientific Procedures) Act, 1986. Following treatment with 2 mg/mL type IIA collagenase (MilliporeSigma), in Ca2+-free standard oocyte saline (SOS) containing 100 mM NaCl, 2 mM KCl, 1 mM MgCl2, and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) (pH 7.6), oocytes were transferred to SOS containing 100 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, and 5 mM Hepes (pH 7.6) for removal of the follicle cell layers (44, 45). cRNA encoding each nAChR subunit and cofactor was prepared from the cDNA construct using the mMESSAGE mMACHINE T7 Ultra Kit (Thermo Fisher Scientific). The cytoplasm of defolliculated oocyte was injected with 50 nL of cRNA solution, where each cRNA was injected into the same concentration (100 ng μL−1). Injected oocytes were incubated in SOS supplemented with 2.5 mM sodium pyruvate, 100 units mL−1 penicillin, 100 μg mL−1 streptomycin, and 20 μg mL−1 gentamycin in holding medium (50% horse serum (Thermo Fisher Scientific) at 16 °C for 3 to 7 days to elute and record of the responses. Voltage-clamp electrophysiology was performed with oocytes clamped at a holding potential E0 of −100 mV. Data were analyzed using Clampfit (Molecular Devices). Oocytes were perfused at 7 to 10 mL·min−1 with SOS.
containing 0.5 μM atropine (A-SOS) to block endogenous muscarinic ACh receptor response (44–46). Stock solutions of neonicotinoids were prepared in dimethyl sulfoxide (DMSO) at 100 mM and diluted with A-SOS to prepare test solutions. DMSO in test solutions <0.1% had no effect on the nAChR responses at this concentration range. ACh was dissolved directly in A-SOS immediately before experiments. When determining concentration–response relationships for ACh and neonicotinoids, responses to 100 μM ACh were first measured by successive applications for 5 s at 3-min intervals to confirm that the responses are stable, prior to applications of these agonists for 5 s at 3-min intervals. At higher concentrations, one oocyte was used to record one response to the neonicotinoids of all of the wild type, *D. melanogaster*, *A. mellifera*, and *B. terrestris* nAChRs tested to prevent the effect of irreversible modulation of the wild-type nAChRs. In the cases of the *D. melanogaster* nAChRs, peak amplitude of the ACh- and neonicotinoid-induced response was normalized to the response to ACh at either 100 μM (Dα1/Dβ1 nAChR), 300 μM (Dα1/Dα2/Dβ1 and Dα1/Dα2/Dβ1/Dβ2 nAChRs), or 300 μM (Dα1/Dβ1/Dβ2 nAChR). In the case of the *A. mellifera* and *B. terrestris* nAChRs, the amplitude of the agonist response to was normalized to the 100 μM ACh-induced response. Experiments were repeated to confirm reproducibility (n = 5, ≥2 frogs).

The concentration–response data were fitted by nonlinear regression using Prism 6 (GraphPad Software) according to the following equation.

\[ Y = \frac{I_{\text{max}}}{1 + 10^{\log(C_0 - X_{\text{H}})}} \]

In Eq. 1, \( I_{\text{max}} \) is normalized maximum response, \( Y \) is normalized response, \( X \) is log [agonist concentration (molar)], and \( n_H \) is the Hill coefficient.

The antagonist effects of neonicotinoids on nAChR were evaluated as follows. After successive applications of 100 μM ACh for 5 s with 3-min interval (training applications), 100 μM ACh was applied for 2 min to oocytes expressing nAChRs. Then, each neonicotinoid was applied for 1 min prior to coapplication with 100 μM ACh for 2 min. The peak amplitude of the responses to ACh in the absence (control, “C”) and presence (treated, “T”) of neonicotinoids was normalized by the mean amplitude of two training ACh responses ([TC1 + TC2]/2). The normalized data for the control [2C/(TC1 + TC2)] and treated responses [2T/(TC1 + TC2)] were compared to evaluate the antagonist actions of the neonicotinoids (SI Appendix, Fig. S7). Experiments were repeated to confirm reproducibility of the antagonist actions of neonicotinoids (n = 5, ≥2 frogs).

**Statistical Analysis.** One-way ANOVA was used to analyze the effects of neonicotinoids on the ACh responses of the *D. melanogaster*, *A. mellifera*, and *B. terrestris* nAChRs expressed in *X. laevis* oocytes, while two-way ANOVA was employed to examine the effect of the R81T mutation on the
agonist activity for each ligand. Any difference between the means considered was analyzed with P values (<0.05) using Prism 6.

**Fly Culture.** *D. melanogaster* flies were raised on cornmeal–agar–yeast medium at 25 °C. UAS-GFP (47) was a gift from Kei Ito, University of Cologne, Cologne, Germany. UAS-mCD8-GFP (#108068), which expresses a green fluorescent protein, was transferred into fertilized eggs maternally expressing Cas9 protein. The flanking gene-specific sequence of the gRNA is indicated by a slash. The 20-bp gene-specific sequence of the gRNA is underlined:

```
D-AGAGGACTGGAAGTACGTTGCCATG/GTAT
''`

The targeting vector was designed such that the site of integration is shown below with the site of integration offset value, 0.123) and then the phylogenetic tree was built with fruit fly strains. We also thank Yoshihiro Fukuta and Daiki Higuchi for assistance; Jun Nakamura and Shinji Kohara for the gift of honeybees and bumblebees, respectively; Tamara Clark for the drawings of fruit fly, honeybee, and bumblebee; and Jun Nakamura for the gift of the photographs of honeybee and bumblebee.

Data Availability. Sequence data are deposited in DNA Data Bank of Japan (accession numbers BCD56239, BCD56240, and BCD56241). All other data are included in the manuscript and SI Appendix.

**ACKNOWLEDGMENTS.** This study was supported by Japan Society for the Promotion of Science (KAKENHI to M.I. (Grant 16K21507), H.T. (Grant 26250001), as well as to K.M. and R.N. (Grant 17H01472). Y.Y. was a recipient of the fellowship from the Japan Society for the Promotion of Science. We thank Kei Ito and Kyoto Stock Center (Drosophila Genetic Resource Center) at Kyoto Institute of Technology for providing us with the fruit fly strains. We also thank Yoshihiro Fukuta and Daiki Higuchi for assistance; Jun Nakamura and Shinji Kohara for the gift of honeybees and bumblebees, respectively; Tamara Clark for the drawings of fruit fly, honeybee, and bumblebee; and Jun Nakamura for the gift of the photographs of honeybee and bumblebee.

**REFERENCES.**

1. K. Matsuda et al., Neonicotinoids: Insecticides acting on insect nicotinic acetylcholine receptors. Trends Pharmacol. Sci. 22, 573–580 (2001).
2. N. Nauen, U. Ebbinghaus-Kintisch, A. Elbert, P. Jeschke, K. Tietjen, “Acetylcholine receptors as sites for developing neonicotinoid insecticides” in Biochemical Sites of Insecticide Action and Resistance, I. Ishaya, Ed. (Springer, 2001), pp. 77–105.
3. P. Jeschke, R. Nauen, M. E. Beck, Nicotinic acetylcholine receptor agonists: A milestone for modern crop protection. Angew. Chem. Int. Ed. Engl. 52, 9464–9465 (2013).
4. J. E. Casida, Neonicotinoids and other insect nicotinic receptor competitive modulators: Progress and prospects. Annu. Rev. Entomol. 63, 125–144 (2018).
5. K. Matsuda, M. Ibara, D. B. Sattelle, Neonicotinoid insecticides: Molecular targets, resistance, and toxicity. Annu. Rev. Pharmacol. Toxicol. 60, 241–255 (2020).
6. M. Rudolf and C. Ellickson, Neonicotinoid insecticide negatively affects wild bees. Nature 521, 77–80 (2015).
7. J. R. Gill, O. Ramos-Rodriguez, N. E. Rainie, Combined pesticide exposure severely affects individual- and colony-level traits in bees. Nature 491, 105–108 (2012).
8. R. P. Whitehorn, S. O’Connor, F. L. Wackers, D. Goulson, Neonicotinoid pesticide reduces bumble bee colony growth and queen production. Science 336, 351–352 (2012).
9. B. A. Woodcock et al., Country-specific effects of neonicotinoid pesticides on honey bees and wild bees. Science 356, 1393–1395 (2017).
10. D. Cresssey, The bitter battle over the world’s most popular insecticides. Nature 551, 156–157 (2018).
11. J. D. Crall et al., Neonicotinoid exposure disrupts bumblebee nest behavior, social networks, and thermoregulation. Science 362, 683–686 (2018).
12. D. Wintermantel et al., Field-level clothianidin exposure affects bumblebees but generally not their pathogens. Nat. Commun. 9, 5446 (2018).
13. J. Lémaître, E. Kuekela, J. Tuomi, S. Juntunen, P. C. Watts, Low dose of neonicotinoid insecticide reduces foraging motivation of bumblebees. Proc. Biol. Sci. 285, 20180506 (2018).
14. C. A. Hallmann, R. P. Popfen, C. A. van Turnhout, H. de Kroon, E. Jongejans, Declines in invertebrate birds are associated with high neonicotinoid concentrations. Nature 511, 341–343 (2014).
15. D. Goulson; 232 signatories, Call to restrict neonicotinoids. Science 360, 973 (2018).
16. Z. Strickland et al., Physiological properties of neuronal nicotinic receptors recovered from the vertebrate j2 subunit and Drosophila un subunits. Eur. J. Neurosci. 6, 869–875 (1994).
17. M. Ibara, S. D. Buckingham, K. Matsuda, D. B. Sattelle, Modes of action, resistance and toxicity of insecticides targeting nicotinic acetylcholine receptors. Curr. Med. Chem. 26, 2925–2940 (2019).
18. M. Ibara, K. Matsuda, Neonicotinoids: Molecular mechanisms of action, resistance and impact on pollinators. Curr. Opin. Insect Sci. 30, 86–92 (2018).
19. G. S. Barbaro, C. Zube, J. Rybak, M. Gauthier, B. Grünewald, Acetylcholine, GABA and glutamate induceionic currents in cultured antennal lobe neurons of the honeybee, Apis mellifera. J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol. 191, 823–836 (2005).
20. G. S. Barbaro, B. Grünewald, S. Paute, M. Gauthier, V. Raymond-Delpech, Study of nicotinic acetylcholine receptors on cultured antennal lobe neurones from adult honeybee brains. Insect Neurosci. 3, 19–29 (2008).
21. P. Déglise, B. Grünewald, M. Gauthier, The insecticide imidacloprid is a partial agonist of the nicotinic receptor of honeybee Kenyon cells. Neurosci. Lett. 321, 13–16 (2002).
22. M. Ibara et al., Actions of imidacloprid, clothianidin and related neonicotinoids on nicotinic acetylcholine receptors of American cockroach neurons and their relationships with insecticidal potencies. J. Toxicol. Sci. 31, 35–40 (2006).
23. L. A. Brown, M. Ibara, S. D. Buckingham, K. Matsuda, D. B. Sattelle, Neonicotinoid insecticides display partial and super agonist actions on native insect nicotinic acetylcholine receptors. J. Neurochem. 99, 608–615 (2006).
24. V. L. Salgado, R. Saar, Desensitizing and non-desensitizing subtypes of α-bungarotoxin-sensitive nicotinic acetylcholine receptors in cockroach neurons. J. Insect Physiol. 50, 867–879 (2004).
25. S. H. Thany, Agonist actions of cloothionin on synaptic and extrasympathetic nicotinic acetylcholine receptors expressed on cockroach sixth abdominal ganglion. Neurotoxicology 30, 1045–1052 (2009).
26. J. Haugstetter, T. Blücher, L. Ellgaard, Identification and characterization of a novel thoredoxin-related transmembrane protein of the endoplasmic reticulum. J. Biol. Chem. 280, 8371–8380 (2005).
27. P. Schloss, H. Betz, C. Schröder, E. D. Gundelfinger, Neuronal nicotinic acetylcholine receptors in Drosophila: Antibodies against an α-like and a non-α subunit recognize the same high-affinity α-bungarotoxin binding complex. J. Neurochem. 57, 1556–1562 (1991).
28. K. Chamaon, K. H. Smalla, U. Thomas, E. D. Gundelfinger, Nicotinic acetylcholine receptors in Drosophila: Three subunits encoded by genomically linked genes can co-assemble into the same receptor complex. J. Neurochem. 80, 149–157 (2001).
29. F. Diao, B. H. White, A novel approach for directing transgenic expression in Drosophila: T2A-Ga4 in-frame fusion. Genetics 190, 1139–1144 (2012).
30. S. Kondo et al., Neurochemical organization of the Drosophila brain visualized by Drosophila  לקרוא Brylinski tagged neurotransmitter receptors. Cell Rep. 30, 284–297 e5 (2020).
31. C. Rezával, T. Nojima, M. C. Neville, A. C. Lin, S. F. Goodwin, Sexually dimorphic octopaminergic neurons modulate female postmating behaviors in Drosophila. Curr. Biol. 24, 725–730 (2014).
32. S. L. Landell, B. Schmitt, H. Betz, D. B. Sattelle, N. S. Miller, Temperature-sensitive expression of Drosophila neuronal nicotinic acetylcholine receptors. J. Neurochem. 68, 1812–1819 (1997).
33. S. Halevi et al., The *C. elegans* ric-3 gene is required for maturation of nicotinic acetylcholine receptors. *EMBO J.* 21, 1012–1020 (2002).
34. S. Eimer et al., Regulation of nicotinic receptor trafficking by the transmembrane Golgi protein UNC-50. *EMBO J.* 26, 4313–4323 (2007).
35. N. Unwin, Y. Fujiyoshi, Gating movement of acetylcholine receptor caught by plunge-freezing. *J. Mol. Biol.* 422, 617–634 (2012).
36. Á. Nemez, M. S. Prevost, A. Menny, P. J. Corringer, Emerging molecular mechanisms of signal transduction in pentameric ligand-gated ion channels. *Neuron* 90, 452–470 (2016).
37. T. Boulin et al., Eight genes are required for functional reconstitution of the *Caenorhabditis elegans* levamisole-sensitive acetylcholine receptor. *Proc. Natl. Acad. Sci. U.S.A.* 105, 18590–18595 (2008).
38. M. J. Palmer et al., Cholinergic pesticides cause mushroom body neuronal inactivation in honeybees. *Nat. Commun.* 4, 1634 (2013).
39. C. Manjon et al., Unravelling the molecular determinants of bee sensitivity to neonicotinoid insecticides. *Curr. Biol.* 28, 1137–1143.e5 (2018).
40. H. C. Godfray et al., A restatement of the natural science evidence base concerning neonicotinoid insecticides and insect pollinators. *Proc. Biol. Sci.* 281, 20140558 (2014).
41. J. T. Kerr et al., CLIMATE CHANGE. Climate change impacts on bumblebees converge across continents. *Science* 349, 177–180 (2015).
42. S. Shimada et al., The mechanism of loop C-neonicotinoid interactions at insect nicotinic acetylcholine receptor α1 subunit predicts resistance emergence in pests. *Sci. Rep.* 10, 7529 (2020).
43. J. Huang, W. Liu, Y. X. Qi, J. Luo, C. Montell, Neuromodulation of courtship drive through tyramine-responsive neurons in the *Drosophila* brain. *Curr. Biol.* 26, 2246–2256 (2016).
44. C. A. Schneider, W. S. Rasband, K. W. Eliceiri, NIH image to ImageJ: 25 years of image analysis. *Nat. Methods* 9, 671–675 (2012).
45. K. Katoh, K. Misawa, K. Kuma, T. Miyata, MAFFT: A novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res.* 30, 3059–3066 (2002).