Article

USE OF PRIMARY CULTURES OF KENYON CELLS FROM BUMBLEBEE BRAINS TO ASSESS PESTICIDE SIDE EFFECTS

Daniel E. Wilson, Rodrigo A. Velarde, and Susan E. Fahrbach
Department of Biology, Wake Forest University, Winston-Salem, NC, USA

Veerle Mommaerts and Guy Smagghe
Department of Crop Protection, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium

Bumblebees are important pollinators in natural and agricultural ecosystems. The latter results in the frequent exposure of bumblebees to pesticides. We report here on a new bioassay that uses primary cultures of neurons derived from adult bumblebee workers to evaluate possible side-effects of the neonicotinoid pesticide imidacloprid. Mushroom bodies (MBs) from the brains of bumblebee workers were dissected and dissociated to produce cultures of Kenyon cells (KCs). Cultured KCs typically extend branched, dendrite-like processes called neurites, with substantial growth evident 24–48 h after culture initiation. Exposure of cultured KCs obtained from newly eclosed adult workers to 2.5 parts per billion (ppb) imidacloprid, an environmentally relevant concentration of pesticide, did not have a detectable effect on neurite outgrowth. By contrast, in cultures prepared from newly eclosed adult bumblebees, inhibitory effects of imidacloprid were evident when the medium contained 25 ppb imidacloprid, and no growth was observed at 2,500 ppb. The KCs of older workers (13-day-old nurses and foragers) appeared to be more sensitive to

Daniel Wilson and Veerle Mommaerts contributed equally to this study.
Grant sponsor: Fund of Scientific Research (FWO-Vlaanderen, Belgium); Grant sponsor: Special Research Fund of Ghent University (BOF-UGent); Grant sponsor: Provost's Office of Wake Forest University.
Correspondence to: Susan E. Fahrbach, Department of Biology, Wake Forest University, Box 7325, Winston-Salem, NC 27109, USA. E-mail: fahrbach@wfu.edu

ARCHIVES OF INSECT BIOCHEMISTRY AND PHYSIOLOGY, Vol. 84, No. 1, 43–56 (2013)
Published online in Wiley Online Library (wileyonlinelibrary.com).
 DOI: 10.1002/arch.21112

imidacloprid than newly eclosed adults, as strong effects on KCs obtained from older nurses and foragers were also evident at 2.5 ppb imidacloprid. In conclusion, primary cultures using KCs of bumblebee worker brains offer a tool to assess sublethal effects of neurotoxic pesticides in vitro. Such studies also have the potential to contribute to the understanding of mechanisms of plasticity in the adult bumblebee brain. © *2013 Wiley Periodicals, Inc.*

Keywords: imidacloprid; Kenyon cells; neonicotinoids; neurite

INTRODUCTION

To find the location of food sources, social bees rely on sensory information that they receive from nest mates and the environment. Processing of this information occurs in the brain and information transfer between the antennal lobes and the protocerebral mushroom bodies (MBs) has been confirmed (Hammer and Menzel, 1998; Thum et al., 2007). In honeybees, which have been most intensively studied, olfactory information received in the antennal lobes is sent via cholinergic projection neurons to the calyces of the MBs; the axon terminals of these projection neurons synapse onto dendrites of the Kenyon cells (KCs) (Kelber et al., 2006). In the MBs, the KCs perform a final integration of olfactory, visual, and gustatory signals (Brandt et al., 2005). In vivo, the MBs of the honeybee brain display considerable structural plasticity: the neuropils associated with the MBs become larger as foraging experience is accumulated (Withers et al., 1993; Durst et al., 1994; Ismail et al., 2006). Flight experience was found to be correlated with changes in dendritic spine morphology of the KCs and with changes in the number and volume of areas of synaptic contacts in the calyces of the MBs (Coss et al., 1980; Krofczik et al., 2008). Several critical studies have indicated that the changes observed in the MBs in foragers reflect foraging experience rather than age (Withers et al., 1993; Farris et al., 2001). Calcium imaging of the brains of honeybees during pairing of an odor with a sucrose reward revealed changes in the pattern of KC activation, a strong indication of functional plasticity that may be linked to the subsequent structural plasticity (Szyszka et al., 2008). Neurotransmitters, including acetylcholine and octopamine, have been demonstrated to modulate odor-based associative learning in honeybees, and it is possible that they regulate experience-dependent structural changes in the MBs (Hammer and Menzel, 1998; Ismail et al., 2006; Gauthier, 2010; Dobrin et al., 2011).

Honeybees and bumblebees are economically significant pollinators in natural and agricultural ecosystems. Bumblebees have a high probability of being exposed to pesticides as they forage (Goulson, 2010). Compounds active at very low concentrations such as the neonicotinoid imidacloprid are of particular concern. Imidacloprid residues have been recovered from honeybee tissues and bee-produced materials such as wax, and have been shown to interfere at low doses (a few parts per billion, ppb) with behaviors essential for foraging including orientation, learning, mobility, navigation, and reflexive extension of the proboscis in response to sugars (reviewed by Blacquière et al., 2012). Mommaerts et al. (2010) reported significant negative effects of oral exposure to pesticides, including imidacloprid, on the survival and foraging performance of bumblebee workers (*Bombus terrestris*) housed in small colonies in a greenhouse. In this study, the effects of imidacloprid were evident at very low doses: 10 and 20 ppb both resulted in sublethal effects, including a reduction in foraging. Exposure of *B. terrestris* colonies in the laboratory to

environmentally relevant concentrations of imidacloprid via contaminated pollen and sugar water resulted in negative effects on colony development and queen production when these colonies were subsequently returned to the field (Whitehorn et al., 2012). Such colony-level effects can have severe consequences for bumblebee populations because, in contrast to honeybees, bumblebee colonies have an annual cycle with only queens overwintering (Heinrich, 1979).

Pollinator populations are currently experiencing a global decline (Potts et al., 2010). Although it is likely that the abundance of pollinators is determined by multiple factors, numerous authors have suggested that pollutants such as pesticides may contribute, possibly importantly, to this decline (Decourtye and Devillers, 2010; Neumann and Carreck, 2010; Kluser et al., 2011). In this context, regulatory agencies are increasingly imposing requirements to assess the sublethal effects of pesticides before approval for field use is granted. Current tests typically cover short periods of exposure and examine only the overall health of the colony or the individual. Such methods may fail to detect subtle effects on behavior caused by direct actions of pesticides on the central nervous system.

Imidacloprid is an agonist of the insect nicotinic acetylcholine receptor (nAChR). This receptor is expressed in many brain regions, including the KCs of the MBs and the antennal lobes of honeybees, sites of intense olfactory information processing (Goldberg et al., 1999; Wüstenberg and Grünewald, 2004; Barbara et al., 2005). This distribution of receptors predicts that imidacloprid and other neonicotinoids could potentially affect the structure and function of neurons in central olfactory pathways.

Cultures of dissociated insect neurons have proved useful for the study of neuronal growth in controlled environments (Thomas et al., 1987; Kirchhof, and Bicker, 1992; Beadle, 2006), and this method has successfully been applied previously in studies of honeybee neurons (Schäfer et al., 1994; Kirchhof and Mercer, 1997; Velarde et al., 2010). Such cultures are often referred to as primary cultures to distinguish them from immortalized neural tumor cell lines, which rarely display a full neuronal phenotype (Murayama et al., 2001). Here, we report on a new bioassay that uses primary cultures of KCs from the brains of adult bumblebees (*Bombus impatiens*) to evaluate the side-effects of environmentally relevant (2.5 ppb) and higher concentrations (up to 2,500 ppb) of imidacloprid. Initially, KCs derived from the brains of newly eclosed workers were cultured and exposed, via the culture medium, to different concentrations of imidacloprid. Three parameters were scored for individual KCs: (i) presence or absence of neurite outgrowth, (ii) the length of the primary neurite, and (iii) the area of the convex polygon encompassed by all branches of the neurite. These experiments were repeated using KCs obtained from age-matched nurses and foragers (13 days old). This permitted us to assess if neuronal sensitivity to imidacloprid depends on age and/or task.

MATERIALS AND METHODS

Insects

Two hives of *B. impatiens* were obtained from Biobest Canada (Leamington, ON, Canada). These hives were maintained in a greenhouse at Wake Forest University in Winston-Salem, NC. The food consisted of sugar water (Biogluc 50%) and pollen grains (collected by honeybees) presented to foragers at a feeding station placed approximately 2 m from the hive entrance. The average temperature in the greenhouse was 23°C; the relative humidity in the room ranged from 31 to 51%.

Imidacloprid

A stock solution of 25 ppm imidacloprid was prepared by dissolving 100 mg imidacloprid (Sigma-Aldrich 37894, St. Louis, MO) in 200 ml culture grade water at room temperature (RT, ~20°C) and diluting it 1/20 in culture grade water. Subsequently, the required working concentrations (2.5, 25, and 2,500 ppb) were prepared using culture grade water immediately prior to their addition to the culture medium, as described in the following sections.

Labeling of Bumblebee Workers

In the evening, hive boxes were temporarily moved into the laboratory so that they could be inspected under dim red light illumination, which bumblebees cannot see (Peitsch et al., 1992). All newly eclosed adult workers present in the hives were labeled on the dorsal thorax with a white paint mark. These young workers are easy to distinguish from older workers because of their pale color. After labeling, the hives were returned to the greenhouse. On each subsequent day, the sugar water dishes at the feeding stations were observed for 30 min at 09:00 h and 18:00 h for the presence of foragers with a white paint mark. These nectar foragers were labeled with a second color of paint (red). In the evening, the hives were again brought into the laboratory and the newest set of pale workers was labeled on the dorsal thorax with a different color for observations at the feeding station on subsequent days. At this time, all workers with a white paint mark observed performing nursing tasks were labeled with a second color (in this case, blue). This procedure was repeated daily using distinct paint colors until two 1-day olds, two nurses, and two foragers of the same age as the nurses were marked per hive. Nontoxic Uni POSCA paints (Mitsubishi Pencil Co. Ltd., Tokyo, Japan) were used for marking bumblebees.

Dissection and Dissociation of the MBs

The procedure was modified from protocols initially developed for use with KCs obtained from *Drosophila melanogaster* (Kraft et al., 1998) and *Apis mellifera* (Schäfer et al., 1994; Velarde et al., 2010). The marked adult bumblebees were collected directly from the hives and cooled briefly on ice until immobilized. The nurses and foragers were collected for dissection at 13 days of age. The head of each bumblebee was removed using a razor blade, and the head capsule was dipped briefly in cold 70% ethanol. The brain was quickly freed from the head capsule using sharp forceps. The MBs were dissected in cold hypertonic L-15 medium (Sigma-Aldrich) supplemented with 4 g glucose, 2.5 g fructose, 44 g sucrose, and 3.3 g proline per 500 ml of medium. Four isolated MBs (two per brain) were transferred to a siliconized microcentrifuge tube (Midsci, St. Louis, MO) containing cold Ringer's solution (135 mM NaCl, 5 mM KCl, 180 mM sucrose, and 20 mM HEPES; all culture grade reagents) and incubated for 2 min on ice. Then, the MBs were incubated at RT in 180 µl 1X trypsin (T-4174, Sigma-Aldrich) for 6 min followed by a 5 min incubation with 180 µl trypsin inhibitor (Type 1-S from Soybean, T-6522 Sigma-Aldrich). After 1 min centrifugation at 800 rpm at 4°C, the supernatant was withdrawn and discarded. Warmed (27°C) supplemented L-15 medium was added, and the MB were dissociated by gentle trituration with the tip of a 200 µl Pipetman (Rainin, Oakland, CA). After a brief waiting period (to allow debris to settle), the cell solution was transferred into a new siliconized

microcentrifuge tube. This procedure was carried out under very clean conditions using sterile tools.

Culture Methods

Round 12-mm glass cover slips coated with poly-D-lysine/laminin (354087, BD Biosciences, San Jose, CA) were placed on the bottom of a 24-well plate (Falcon multiwell tissue culture treated plates, BD, Franklin Lake, NJ). The cell solutions containing the KCs were added to the wells, and the plate was transferred to a 27°C incubator. After allowing 5 min for settling, the cover slips were gently washed with warmed supplemented L-15 medium. The cover slips were then incubated at 27°C for 24 h with warmed supplemented L-15 medium containing 10 μl Gibco B-27 (Life Technologies, Grand Island, NY) and 10 μl of 4 ppb 20-hydroxyecdysone (20E; H-5142, Sigma-Aldrich). The hormone 20E was added because previous studies showed that this hormone promotes neurite outgrowth in cultured honeybee neurons (Velarde et al., 2010). The final medium also contained different concentrations of imidacloprid (0, 2.5, 25, and 2,500 ppb). Cultures exposed to 20E and 0 ppb imidacloprid are described as 20E-only controls in the following presentation of our data.

Immunolabeling and Imaging

After 24 h, the culture medium was removed and all cultures were fixed for 30 min with 4% w/v paraformaldehyde (Sigma-Aldrich) in PBS (0.01 M, pH 7.4). The cover slips were washed three times in PBS and incubated with 5% normal goat serum for 1 h to block nonspecific labeling. After blocking, the cover slips were incubated overnight at 4°C with the primary antibody (1:800 rabbit anti-HRP; P7899, Sigma-Aldrich). This antibody labels a cell surface glycoprotein previously shown to be expressed by many populations of insect neurons (Jan and Jan, 1982); the immunolabeling produced by this antibody therefore serves as a general neuronal marker, ensuring that only neurons are evaluated. Following the incubation with the primary antibody, cover slips were washed three times at 5 min intervals with PBS. Cover slips were then incubated with goat anti-rabbit Alexa Fluor 488 (1:500 dilution; Life Technologies) for 2 h. After two final washes in PBS, the cover slips were mounted onto glass microscope slides using Vectashield medium containing the nuclear marker DAPI (Vector Laboratories, Burlingame, CA).

Stacks of images of cultured KCs were acquired using a Zeiss AxioObserver inverted microscope equipped for epifluorescent illumination. This microscope is controlled with a Mac 6000 Controller System from Ludl Electronic Products, Ltd. (Hawthorne, NY), permitting acquisition of optical sections with minimal photobleaching. A 20X Plan Neofluar objective with a numerical aperture of 0.5 was used, and images were captured with a Hamamatsu Orca C4742-80-12AG digital camera. Images were acquired as bitmap files using the default settings of Improvision Volocity® software (Perkin-Elmer, Waltham, MA) and opened using the 2009 edition of the ZEN lite imaging software (http://microscopy.zeiss.com/microscopy/en_de/products/software/zen-lite.html).
Because the first processes to grow in these primary cultures could not be definitively identified as axons or dendrites, they are hereafter referred to as neurites. Three parameters related to neurites were evaluated: presence or absence of extended processes (neurite formation), the length of the primary neurite (μm), and the area of the smallest convex polygon area including all branches of all neurites (μm^2), which is a measure of branching complexity. Most KCs grown under these conditions extend only a single

neurite, which is by default defined as the primary neurite; in cases in which more than a single neurite was extended, the longest neurite was defined as the primary neurite. Cultured neurons were selected for evaluation using a straight line transect survey method with a sampling belt one field of view wide. At the start of each transect (imaginary straight line across the cover slip), the objective was positioned at one edge of the round 12-mm cover slip. The observer then acquired an image stack for every neuron in the field of view. The stage was then moved to the next point in the transect, and an image stack was acquired for every neuron in the new field of view. This process was repeated until image stacks had been acquired for the first 25 neurons encountered on a cover slip. Note that neurons with overlapping processes were excluded from the analysis to ensure that neurites were not assigned to the wrong soma (neuronal cell body). Because our individual cultures were by design of very low density, it was rarely necessary to exclude a neuron for this purpose. A typical cover slip prepared using the method described above contains only several thousand KCs. Multiple cultures from different brains could be prepared in a single morning, and all of the data presented here were obtained from cultures prepared from different pairs of bumblebees and established within a 2-week period, with multiple concentrations of imidacloprid tested on each culturing day.

Statistics

For each parameter (neurite formation, neurite length, and area of the convex polygon), two nonoverlapping, independent samples of 25 KCs were analyzed per imidacloprid concentration and per age/behavioral status. The following samples were obtained for analysis: from Colony 1, two 1-day olds, two 13-day-old nurses, and two 13-day-old foragers; from Colony 2, two 1-day olds, two 13-day-old nurses, and two 13-day-old foragers. The MBs from each pair of bumblebees of the same age/behavioral status/colony source were pooled to yield four cultures per pair. Each of the resulting four cultures was assigned to a different treatment: 20E only, 2.5 ppb imidacloprid, 25 ppb imidacloprid, or 2,500 imidacloprid. Therefore, a total of 24 cultures were analyzed for this study. Because there is no evidence that the growth of individual, nonconfluent KCs is influenced by the presence of other KCs on the cover slip, we treated each KC as an independent data point and then summarized the data per cover slip so as not to artificially inflate the sample size. For neurite length and the area of the convex polygon, a normal distribution was confirmed by the Kolmogorov–Smirnov test ($P = 0.05$). Then, unless stated otherwise, the data (mean ± standard deviation, STDV) were analyzed by one-way analysis of variance (ANOVA) followed by the post hoc Tukey–Kramer test ($\alpha = 0.05$) using SPSS v. 16.0. A Student's *t*-test was used for pairwise comparisons of the effect of different concentrations of imidacloprid on the length of the primary neurite and the area of the convex polygon defined by all neurites. We did not analyze our data for the effect of colony given that only two source colonies reared in the same greenhouse were used in these initial studies, and therefore our replicates are best viewed as technical replicates despite the large number of individual KCs that were evaluated. We did not assess differences in responses at the level of individual bumblebees because each cover slip represented a sample of the pooled population of KCs from a matched pair of bumblebees from the same colony. Our primary focus was on the effects of different concentrations of imidacloprid.

RESULTS

Effect of Imidacloprid on KC Growth in 1-Day-Old Workers

Our procedure for short-term primary culture of adult KCs strips these neurons of their dendrites and axons. This permits our cultures to be used as a bioassay for neurite regeneration. Examples of differential responses of cultured bumblebee KCs to a broad range of concentrations of imidacloprid are shown in Figure 1. Compared with the control cultures, KCs from 1-day olds exposed to the highest dose of imidacloprid (2,500 ppb) showed an inhibition of all growth within the 24-h period of observation (0% exhibiting growth). Exposure of KCs to 2.5 ppb and 25 ppb imidacloprid did not affect the percentage of sampled KCs per culture exhibiting growth: 67 ± 4% and 60 ± 17%, compared with 70 ± 2% in the control, 20E-only cultures without imidacloprid (Fig. 2A).

Effect on Primary Neurite Length and Convex Polygon Area of Neurites in 1-Day-Old Workers

In 1-day-old workers, the mean length of the primary neurite following exposure to 2.5 ppb and 25 ppb imidacloprid was not significantly different ($P > 0.05$) than that of 20E-only controls (Fig. 2B). As previously noted, KCs exposed to 2,500 ppb imidacloprid did not show neurite growth (Fig. 2B), and hence this parameter could not be scored for this group.

The area of the convex polygon covered by all neurites produced by an individual KC did not differ ($P > 0.05$) for the cells exposed to 2.5 ppb imidacloprid and the 20E-only controls. There was also no effect on the area of the convex polygon at exposures to 25 ppb imidacloprid. However, the area of the convex polygon encompassed by the growing neurites was significantly ($P < 0.05$) smaller in the 2,500 ppb cultures than in the 20E-only controls (Fig. 2C).

The Influence of Experience and Behavior on the Sensitivity of KCs to Imidacloprid

The percentage of cultured KCs showing growth after exposure to 2.5 ppb imidacloprid was lower in both nurses (20 ± 0%) and foragers (4 ± 6%) than in 1-day olds obtained from the same two colonies (Fig. 3A). The two higher concentrations of imidacloprid were also associated with a reduction in the percentage of KCs extending a neurite. The highest concentration (2,500 ppb) resulted in growth in 26 ± 9% of sampled KCs for nurses and in 6 ± 3% for foragers, compared with 76 ± 1% in the imidacloprid-free controls.

The length of the primary neurite was reduced ($P < 0.05$) in both nurse- and forager-derived KCs exposed to 2.5 ppb imidacloprid (Fig. 3B). The length of the primary neurite was also reduced after exposure to the 2,500 ppb imidacloprid: 5 ± 1 μm for nurses and 1 ± 0 μm for foragers. Note that these values represent the growth of a very small number of neurites in a small number of KCs in the imidacloprid-containing cultures, and these calculations include many zero values (no growth).

The mean area of the convex polygon enclosing all neurites was lower in nurses and foragers exposed to 2.5 ppb imidacloprid than in 20E-only controls (Fig. 3C). Increasing the concentration of imidacloprid to 25 ppb caused a further decrease of the mean convex polygon area of all processes in both nurses (0 ± 0 μm^2) and foragers (1 ± 0 μm^2). Again, these calculations should be interpreted in a qualitative fashion only because they include many zero values.

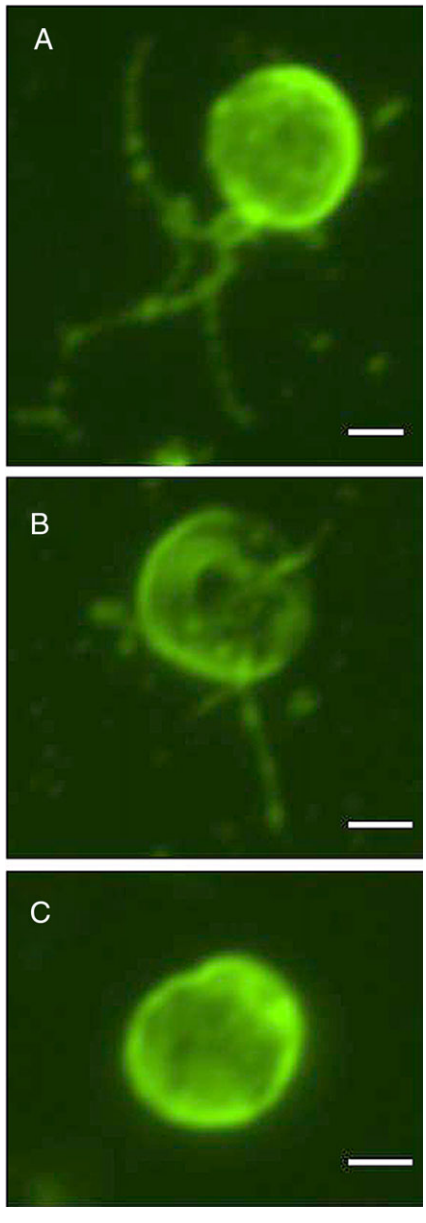

Figure 1. Examples of the impact of imidacloprid treatment on neurite growth of KCs derived from 1-day-old *Bombus impatiens* workers. (A) KCs exposed to 20-hydroxyecdysone only (20E; control), (B) KCs exposed to 20E plus 2.5 ppb imidacloprid showing reduced neurite growth, and (C) KCs exposed to 20E plus 2,500 ppb imidacloprid showing inhibition of growth. Scale bar is 10 μm.

DISCUSSION

The present study describes a novel bioassay using KCs dissected from adult bumblebee brains that can be used to assess the neurotoxicity of compounds such as the neonicotinoid imidacloprid. This assay can be completed in 1–2 days. The first application of this method

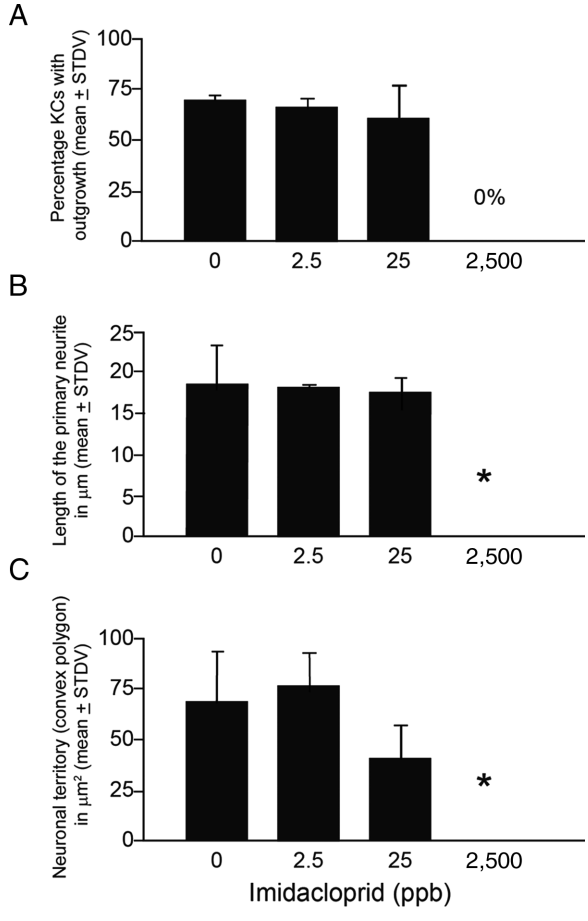

Figure 2. Overview of the effect of different concentrations of imidacloprid on the percentage of KCs exhibiting growth (A), on the length (µm) of the primary neurite (B), and on the area of the convex polygon (μm^2) defined by all neurite branches (C) on KCs from 1-day-old (newly eclosed) *Bombus impatiens* workers. Data are based on the analysis of 25 KCs per cover slip, two independently prepared cover slips (different popuplations of KCs from different bumblebees) per data point. The length of the primary neurite and the area of the convex polygon are expressed as mean values ± STDV (standard deviation). For these measures, statistical analyses with one-way ANOVA across imidacloprid concentration revealed an effect of imidacloprid concentration on the length of the primary neurite ($F = 25.103$, df = 7, $P = 0.005$) and on the area of the convex polygon ($F = 7.285$, df = 7, $P = 0.042$). Values indicated by asterisk (*) are significantly different after post hoc Tukey–Kramer tests ($\alpha = 0.05$).

revealed a possible difference in sensitivity toward imidacloprid between newly eclosed bumblebee workers and 13-day-old nurses and foragers, with the older workers showing greater inhibition of neuronal growth relative to controls than younger workers from the same colony. It is possible that the presence of imidacloprid in the cultures influenced neuronal survival in a dose-dependent fashion, but our neurite-focused analysis does not permit us to assess survival as we use a neuron-specific cell surface marker that detects living neurons. There were no obvious treatment-dependent differences in the density of neurons in our cultures.

We selected KCs for this project because the MBs are involved in processing of olfactory and visual information highly relevant for foraging, and because these neurons can

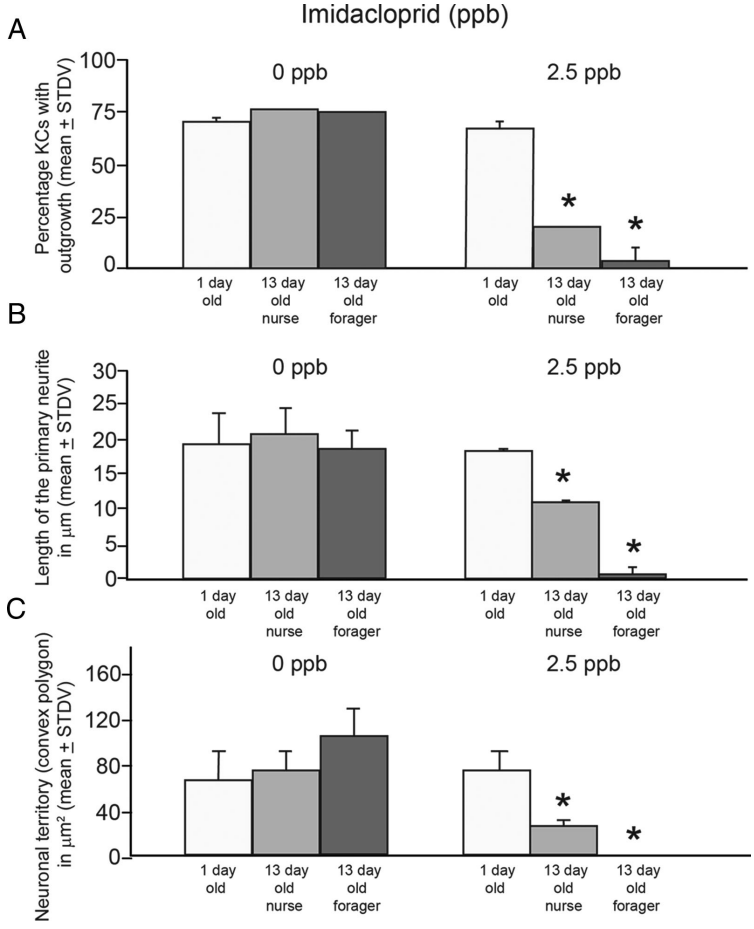

Figure 3. Comparison of the effect of 2.5 ppb imidacloprid on the percentage of KCs extending a neurite (A), on the length (µm) of the primary neurite (B), and on the area of the convex polygon (μm^2) defined by all neurites (C) for 1-day olds, 13-day-old nurses, and 13-day-old foragers of *Bombus impatiens* workers. Data are based on the analysis of 50 KCs per data point. The length of the primary neurite and the area of the convex polygon are expressed as mean values ± STDV (standard deviation). The Student's *t*-test for independent samples used to compare length of the primary neurite for 1-day olds ($t = 0.128$, df = 2, $P = 0.454$), for nurses ($t = 3.286$, df = 2, $P = 0.041$), and for foragers ($t = 9.211$, df = 2, $P = 0.006$), and for the area of the convex polygon defined by all neurites for 1-day olds ($t = 0.389$, df = 2, $P = 0.367$), for nurses ($t = 2.223$, df = 2, $P = 0.031$), and for foragers ($t = 4.114$, df = 2, $P = 0.011$). All treatment groups were compared with their age-matched 20E-only controls. Asterisk (*) indicates values that are significantly different ($P < 0.05$).

be cultured using relatively simple techniques. In addition, this population of central neurons is characterized by substantial dendritic growth and the formation of new synapses during adult life in many hymenopteran species, including bees, wasps, and ants (e.g. Farris et al., 2001; Seid et al., 2005; Molina and O'Donnell, 2008). We want to underline here that we hypothesize that a possible sublethal effect of exposure to neurotoxic pesticides might not be frank brain degeneration, which would likely cause death, but rather a diminished capacity for plasticity. Similar arguments have been developed regarding the effects of neurotoxins on the developing mammalian system (Wallace et al., 2003). Reduced brain plasticity in social insects might in turn reduce the behavioral plasticity required to support normal colony development.

The toxicity and behavioral effects of pesticides such as imidacloprid have been extensively studied (reviewed by Blacquière et al., 2012). Greenhouse experiments that exposed bumblebee hives to sugar water contaminated with imidacloprid revealed that the no-effect concentration (NOEC) for effects on behavior, colony performance, and nest development was ≤ 2.5 ppb (Mommaerts et al., 2010). Studies on honeybees have also reported sublethal behavioral effects at ppb level (Blacquière et al., 2012; Smagghe et al., 2012). Somewhat surprisingly, the KCs of newly eclosed bumblebee workers exposed to 2.5 ppb imidacloprid did not show altered phenotype as evidenced by KC growth, the length of the primary neurite, and a measure of neurite branching (polygon convex area). Higher doses of imidacloprid, however, significantly disrupted KC growth in newly eclosed workers. Growth of neurites was affected for all doses tested in 13-day-old nurse and forager bumblebees, including the lowest. Because our studies only assessed the impact of a 24-h exposure, we cannot rule out the possibility of recovery of growth at later time points.

A correlation between the observed effects of the present study and behavioral effects is difficult. In the present bioassay, individual cells were directly exposed in culture to the full dose of the putative neurotoxin. This is not true for the natural situation in which both metabolic and biological barriers (including the epithelium of the insect gut, and the blood–brain barrier) modulate the concentration of pesticide to which brain neurons are exposed. Although imidacloprid is quickly metabolized by honeybees, a study has demonstrated translocation to various body parts subsequent to ingestion (Suchail et al., 2004a, b). In this study, levels of imidacloprid and its main metabolites in the head were always ≤5% of 100 μg/kg of the initial ingested dose (Suchail et al., 2004a). This is true for an acute exposure, but under field conditions accumulation is possible (see for review Blacquière et al., 2012). Therefore, we speculate that the exposures achieved in the present study are relevant. Our results therefore suggest that the youngest workers in a bumblebee colony are more resistant to imidacloprid than older workers, but that higher doses impact all ages of workers.

At present, the factors that account for the age difference in the response of bumblebees to imidacloprid are unknown. We note that age-dependent differences in behavior and brain plasticity have been previously reported in honeybees. For example, very young honeybees (4–7 days old) required fewer trials than older bees (8–10 days old) to achieve habituation of the proboscis extension reflex (Guez et al., 2001). Structural differences in the MBs, both in terms of dendritic morphology and synaptic organization, have been reported to distinguish young and old honeybees (Coss et al., 1980; Krofczik et al., 2008). The present results suggest that age rather than behavioral status may be a better predictor of responses to imidacloprid, as the KCs of age-matched nurse and forager bumblebees responded similarly to treatment with the pesticide. This conclusion, however, is based on a small sample and should be replicated.

Exposure of KCs obtained from 1-day-old bumblebees to 2.5 ppb imidacloprid did not affect any aspect of neurite growth measured in this study, while in nurses and foragers, the three measured parameters were affected at 2.5 ppb imidacloprid, and at higher concentrations (25 ppb and 2,500 ppb), neurite growth and branching were almost nil. It is therefore possible that the ability to integrate multisensory cues is decreased in older workers exposed to ppb imidacloprid concentrations. This is in accord with the finding that chronic oral exposure to 48 ppb imidacloprid reduced the learning capacity of winter honeybees in laboratory assays of olfactory association learning (Decourtye et al., 2001, 2003, 2004; Lambin et al., 2001). Pesticide-mediated interference with visual orientation has also been documented after exposure to a range of sublethal imidacloprid doses (Bortolotti et al., 2003; Ramirez-Romero et al., 2005; Yang et al., 2008).

In summary, medium-to-low concentrations of imidacloprid inhibited the growth of neurites by bumblebee KCs in primary culture. The same bioassay can be adapted to investigate the neurotoxic effects of other environmental contaminants. In contrast to time-consuming methods for reconstruction of dendrites in intact brains that require specialized equipment and considerable expertise (i.e. serial transmission electron microscopy, Golgi impregnation), primary cultures offer a convenient method with a rapid turn-around. Future studies could use the in vitro approach described here as a screen to select treatment groups for in vivo analysis. We predict that many compounds that affect complex behaviors dependent upon integration of sensory cues, such as foraging, will be shown to inhibit KC growth in vitro. As previously noted, nAChRs are the primary targets of imidacloprid and other neonicotinoids and nAChR expression has been reported in other brain regions, including the antennal lobes (Thany et al., 2005). The bioassay described here may also prove useful in the study of antennal lobe neurons of imidacloprid-exposed bumblebee workers of different ages and/or task specialization. Similar studies could also be performed in honeybees, another pollinator impacted by the widespread agricultural use of neonicotinoids (Blacquière et al., 2012). At present, the effects of exposure to imidacloprid on neurite outgrowth apparent in vitro cannot be directly related to changes in vivo, but our results suggest that direct cellular actions of neonicotinoids on brain neurons should be considered as an explanation for the behavioral effects of these compounds on pollinators.

LITERATURE CITED

Barbara GS, Zube C, Rybak J, Gauthier M, Grünenwald B. 2005. Acetylcholine, GABA and glutamate induce ionic currents in cultured antennal lobe neurons of the honeybee, *Apis mellifera*. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 191:823–836.

Beadle DJ. 2006. Insect neuronal cultures: an experimental vehicle for studies of physiology, pharmacology and cell interactions. Invert Neurosci 6:95–103.

Blacquière T, Smagghe G, van Gestel CAM, Mommaerts V. 2012. Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment. Ecotoxicology 21:973–992.

Bortolotti L, Montanari R, Marcelino J, Medrzycki P, Maini S, Porrini C. 2003. Effect of sub-lethal imidacloprid doses on the homing rate and foraging activity of honey bees. Bull Insectol 56:63–67.

Brandt R, Rohlfing T, Rybak J, Krofczik S, Maye A, Westerhoff M, Hege HC, Menzel R. 2005. Three-dimensional average-shape atlas of the honeybee brain and its applications. J Comp Neurol 492:1–19.

Coss RG, Brandon JG, Globus A. 1980. Changes in morphology of dendritic spines on honeybee calycal interneurons associated with cumulative nursing and foraging experiences. Brain Res 192:49–59.

Decourtye A, Devillers J. 2010. Ecotoxicity of neonicotinoid insecticides to bees. In: Thany SH, editor. Insect nicotinic acetylcholine receptors, 1st ed. New York: Springer. p 85–95.

Decourtye A, Devillers J, Genecque E, Le Menach K, Budzinski H, Cluzeau S, Pham Delègue MH. 2001. Impairment of olfactory learning performances in the honey bee after long term ingestion of imidacloprid. In: Belzunces LP, Pellissier C, Lewis GB, editors. *Hazard of pesticides to bees*. Paris: INRA. p. 113–117.

Decourtye A, Lacassie E, Pham-Delègue MH. 2003. Learning performances of honeybees (*Apis mellifera* L.) are differentially affected by imidacloprid according to the season. Pest Manag Sci 59:269–278.

Decourtye A, Devillers J, Cluzeau S, Charreton M, Pham-Delègue MH. 2004. Effects of imidacloprid and deltamethrin on associative learning in honeybees under semi-field and laboratory conditions. Ecotoxicol Environ Saf 57:410–419.

Dobrin SE, Herlihy JD, Robinson GE, Fahrbach SE. 2011. Muscarinic regulation of Kenyon cell dendritic arborizations in adult worker honey bees. Arthropod Struct Dev 40:409–419.

Durst C, Eichmüller S, Menzel R. 1994. Development and experience lead to increased volume of subcompartments of the honeybee mushroom body. Behav Neural Biol 62:259–263.

Farris SM, Robinson GE, Fahrbach SE. 2001. Experience- and age-related outgrowth of intrinsic neurons in the mushroom bodies of the adult worker honeybee. J Neurosci 21: 6395–6404.

Gauthier M. 2010. State of the art on insect nicotinic acetylcholine receptor function in learning and memory. Adv Exp Med Biol 683:97–115.

Goldberg F, Grünewald B, Rosenboom H, Menzel R. 1999. Nicotinic acetylcholine currents of cultured Kenyon cells from the mushroom body of the honeybee *Apis mellifera*. J Physiol 514: 759–768.

Goulson D. 2010. Bumblebees behaviour, ecology and conservation. New York: Oxford University Press. p 317.

Guez D, Suchail S, Gauthier M, Maleszka R, Belzunces LP. 2001. Contrasting effects of imidacloprid on habituation in 7- and 8-day-old honeybees (*Apis mellifera*). Neurobio Learn Mem 76: 183–191.

Hammer M, Menzel R. 1998. Multiple sites of associative odor learning as revealed by local brain microinjections of octopamine in honeybees. Learn Mem 5:146–156.

Heinrich B. 1979. Bumblebee economics. London: Harvard University Press. p 245.

Ismail N, Robinson GE, Fahrbach SE. 2006. Stimulation of muscarinic receptors mimics experience-dependent plasticity in the honey bee brain. Proc Natl Acad Sci USA 103:207–211.

Jan LY, Jan YN. 1982. Antibodies to horseradish peroxidase as specific neuronal markers in *Drosophila* and in grasshopper embryos. Proc Natl Acad Sci USA 79:2700–2704.

Kelber C, Rossler W, Kleineidam CJ. 2006. Multiple olfactory receptor neurons and their axonal projections in the antennal lobe of the honeybee *Apis mellifera*. J Comp Neurol 496:395–405.

Kirchhof B, Bicker G. 1992. Growth properties of larval and adult locust neurons in primary cell culture. J Comp Neurol 323:411–422.

Kirchhof BS, Mercer AR. 1997. Antennal lobe neurons of the honeybee *Apis mellifera* express a D2-like dopamine receptor *in vitro*. J Comp Neurol 383:189–198.

Kluser S, Neumann P, Chauzat M-P, Pettis JS. 2011. UNEP emerging issues: global honey bee colony disorder and other threats to insect pollinators. Available at http://www.unep.org (accessed July 1, 2011).

Kraft R, Levine RB, Restifo LL. 1998. The steroid hormone 20-hydroxyecdysone enhances neurite growth of *Drosophila* mushroom body neurons isolated during metamorphosis. J Neurosci 18:8886–8899.

Krofczik S, Khojasteh U, Hempel de Ibarra, N, Menzel R. 2008. Adaptation of microglomerular complexes in the honeybee mushroom body lip to manipulations of behavioral maturation and sensory experience. Dev Neurobiol 68:1007–1017.

Lambin M, Armengaud C, Raymond S, Gauthier M. 2001. Imidacloprid- induced facilitation of the proboscis extension reflex habituation in the honeybee. Arch Insect Biochem Physiol 48:129–134.

Molina Y, O'Donnell S. 2008. Age, sex, and dominance-related mushroom body plasticity in the paperwasp *Mischocyttarus mastigophorus*. Dev Neurobiol 68:950–959.

Mommaerts V, Reynders S, Boulet J, Besard L, Sterk G, Smagghe G. 2010. Risk assessment for side-effects of neonicotinoids against bumblebees with and without impairing foraging behaviour. Ecotoxicology 19:207–215.

Murayama K, Singh NN, Helmrich A, Barnes DW. 2001. Neural cell lines. In: Federoff S, Richardson A, editors. Protocols for neural cell culture, 3rd ed. Totowa, NJ: Human Press, Inc. p. 219–228.

Neumann P, Carreck NL. 2010. Honey bee colony loss. J Apic Res 49:1–6.

Peitsch D, Fietz A, Hertel H, Souza J de, Ventura DF, Menzel R. 1992. The spectral input systems of hymenopteran insects and their receptor-based colour vision. J Comp Physiol A 170:23–40.

Potts SG, Biesmeijer JC, Kremen C, Neumann P, Schweiger O, Kunin WE. 2010. Global pollinator declines: trends, impacts and drivers. Trends Ecol Evol 25:345–353.

Ramirez-Romero R, Chaufaux J, Pham-Delegue MH. 2005. Effects of Cry1Ab protoxin, deltamethrin and imidacloprid on the foraging activity and the learning performances of the honeybee *Apis mellifera*, a comparative approach. Apidologie 36:601–611.

Schäfer S, Rosenboom H, Menzel R. 1994. Ionic currents of Kenyon cells from the mushroom body of the honeybee. J Neurosci 14:4600–4612.

Seid MA, Harris KM, Traniello JF. 2005. Age-related changes in the number and structure of synapses in the lip region of the mushroom bodies in the ant *Pheidole dentata*. J Comp Neurol 488:269–277.

Smagghe G, Mommaerts V, Blacquière, T. 2012. Effect of neonicotinoids on pollinators: global adequate risks assessment crucial. Comment for "Field Research on Bees Raises Concern About Low-Dose Pesticides". Science 335:1555. Available at http://comments.sciencemag.org/content/10.1126/science.335.6076.1555.

Suchail S, De Sousa G, Rahmani R, Belzunces LP. 2004a. In vivo distribution and metabolisation of C-14-imidacloprid in different compartments of *Apis mellifera* L. Pest Manag Sci 60:1056–1062.

Suchail S, Debrauwer L, Belzunces LP. 2004b. Metabolism of imidacloprid in *Apis mellifera*. Pest Manag Sci 60:291–296.

Szyszka P, Galkin A, Menzel R. 2008. Associative and non-associative plasticity in Kenyon cells of the honeybee mushroom body. Front Syst Neurosci 2:1–10.

Thany SH, Crozatier M, Raymond-Delpech V, Gauthier M, Lenaerts, G. 2005. Apisα2, Apisα7–1 and Apisα7–2: three new neuronal nicotinic acetylcholine receptor α-subunits in the honeybee brain. Gene 344:125–132.

Thomas WE, Jordan FL, Townsel JG. 1987. The status of the study of invertebrate neurons in tissue culture – phylum Arthropoda. Comp Biochem Physiol A 87:215–222.

Thum AS, Jenett A, Ito K, Heisenberg M, Tanimoto H. 2007. Multiple memory traces for olfactory reward learning in *Drosophila*. J Neurosci 27:11132–11138.

Velarde RA, Wilson DE, Fahrbach SE. 2010. Developmental hormone signals regulate neuronal morphology in adult worker honey bees. Program No. 513.2. 2010 Neuroscience Meeting Planner. San Diego, CA: Society for Neuroscience. Online.

Wallace CS, Reitzenstein J, Withers GS. 2003. Diminished experience-dependent neuroanatomical plasticity: evidence for an improved biomarker of subtle neurotoxic damage to the developing rat brain. Environ Health Perspect 111:1294–1298.

Whitehorn PR, O'Connor S, Wäckers FL, Goulson D. 2012. Neonicotinoid pesticide reduces bumble bee colony growth and queen production. Science 336:351–352.

Withers GS, Fahrbach SE, Robinson GE. 1993. Selective neuroanatomical plasticity and division of labour in the honeybee. Nature 364:238–240.

Wüstenberg DG, Grünewald B. 2004. Pharmacology of the neuronal nicotinic acetylcholine receptor of cultured Kenyon cells of the honeybee, *Apis mellifera*. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 190:807–821.

Yang EC, Chuang YC, Cheng YL, Chang H. 2008. Abnormal foraging behavior induced by sublethal dosage of imidacloprid in the honey bee (Hymenoptera: Apidae). J Econ Entomol 101: 1743–1748.