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Review

A potential link among biogenic amines-based pesticides, learning and memory, and colony collapse disorder: A unique hypothesis

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ABSTRACT

Pesticides are substances that have been widely used throughout the world to kill, repel, or control organisms such as certain forms of plants or animals considered as pests. Depending on their type, dose, and persistence in the environment, they can have impact even on non-target species such as beneficial insects (honeybees) in different ways, including reduction in their survival rate and interference with their reproduction process. Honeybee *Apis mellifera* is a major pollinator and has substantial economical and ecological values. Colony collapse disorder (CCD) is a mysterious phenomenon in which adult honeybee workers suddenly abandon from their hives, leaving behind food, brood, and queen. It is lately drawing a lot of attention due to pollination crisis as well as global agriculture and medical demands. If the problem of CCD is not resolved soon enough, this could have a major impact on food industry affecting world's economy a big time. Causes of CCD are not known. In this overview, I discuss CCD, biogenic amines-based-pesticides (neonicotinoids and formamidines), and their disruptive effects on biogenic amine signaling causing olfactory dysfunction in honeybees. According to my hypothesis, chronic exposure of biogenic amines-based-pesticides to honeybee foragers in hives and agricultural fields can disrupt neural cholinergic and octopaminergic signaling. Abnormality in biogenic amines-mediated neuronal signaling impairs their olfactory learning and memory, therefore foragers do not return to their hive – a possible cause of CCD. This overview is an attempt to discuss a hypothetical link among biogenic amines-based pesticides, olfactory learning and memory, and CCD.

1. Introduction

Honeybees belong to the insect order Hymenoptera. The Western (European) honeybee *Apis mellifera* (genus *Apis* and specie *mellifera*) is the most commonly adaptable species and best known among all insects. Worker honeybees need to forage for a wide diversity of pollen and nectar to raise a healthy brood in the hive and maintain strong immune systems. The clustering behavior and ability to regulate the temperature within the hive irrespective of the external temperature enable honeybees to colonize through a wide variety of environments. Not all insect-dependent pollination is provided by honeybees *A. mellifera*, but the ability to easily move and manage makes them the most economically valuable pollinator of agricultural crops worldwide (vanEngelsdorp et al., 2008; Gallai et al., 2009; vanEngelsdorp and Meixner, 2010). Moreover, honeybee products such as honey, propolis, royal jelly, bee wax, and bee venom offer potential therapeutic importance (Cooper, 2007; Farooqui and Farooqui, 2010, 2011, 2012).

Colony collapse disorder (CCD) is characterized by the rapid decline of the adult bee population, leaving the brood and the queen poorly or completely unattended in the hive. Without the adult bees, colonies eventually collapse because there is no one to provide food and maintain the hive. Several reports have been published on CCD (Cox-Foster et al., 2007; vanEngelsdorp et al., 2007, 2008, 2009; vanEngelsdorp and Meixner, 2010; Bromenshenk, 2010; Bromenshenk et al., 2010; Biesmeijer et al., 2006; Oldroyd, 2007; Palacios et al., 2008; Blanchard et al., 2008; Johnson et al., 2009a; Highfield et al., 2009; VanEngelsdorp et al., 2010; Mullin et al., 2010; Williams et al., 2010; Ratnieks and Carreck, 2010; Wu et al., 2011; Core et al., 2012; Henry et al., 2012; Belzunces et al., 2012; Pareja et al., 2011; Di Prisco et al., 2011; Nazzi et al., 2012). Researchers have been struggling for years to explain CCD, but it remains unknown whether CCD is governed by one specific factor or due to the synergistic action of several factors. If the cause of CCD is not resolved soon enough then this could have a major impact on the world's economy.

My hypothesis is that the chronic exposure (low-dose, overtime) of biogenic amines-based-pesticides (neonicotinoids and formamidines) to honeybees disrupts neuronal cholinergic and octopaminergic signaling and produces excessive reactive oxygen species (ROS) and reactive nitrogen species (RNS) that react with macromolecules and interfere with mitochondrial respiratory chain and mitochondrial Ca^{2+} metabolism, contributing to a deficiency of

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neuronal energy. Oxidative stress impairs cognitive behavior, including olfactory learning and memory, affecting orientation and navigation abilities, therefore, honeybees fail to return to their hives. In this review, I discuss CCD, biogenic amines, biogenic amines-based pesticides and signaling, and a hypothetical interrelated link among pesticides, olfactory learning and memory and CCD.

2. CCD

The term CCD was first introduced to the occurrence of severe abrupt losses in European (Western) worker honeybee *A. mellifera* colonies in North America in late 2006. Since then several substantial drops in managed population of Western honeybee colonies have been noticed throughout North America, in several European countries, portions of Canada, Middle East and Japan. In CCD the remaining honeybee population in hive consists of a queen, sudden inexplicable reduction in workers, and the presence of capped brood with plenty of food stores (honey and pollen) left in colonies. This suggests that major loss of adult workers from hive is not due to the lack of food. One of the oddest of symptoms of CCD is the lack of dead bees of workers being found in or around the hive, speculating that death takes place in the field. Large-scale losses of honeybees are not unusual. In past (1960–1970s), honeybee population has been wiped out due to extreme weather, pesticide exposure, and pest infestation (Oertel, 1965; Foote, 1966; Williams and Kauffeld, 1974; Kauffeld et al., 1976; Olley, 1976; Roberge, 1978). However, CCD differs from past colony losses because it is rapid, more severe, and results in complete loss of foragers with few or no dead bees in or around the hive (Table 1).

2.1. Theories of CCD

Several theories have been suggested for CCD. These theories include infection caused by a microsporidium parasite *Nosema ceranae* or *Nosema apis* (Cox-Foster et al., 2007; Higes et al., 2007, 2008a,b, 2009); varroa mites-*Varroa destructor* injuring both adult honeybees and brood by mite–virus association synergistically reducing host immunity and inducing virus replication (Boecking and Genersch, 2008; Bacandritsos et al., 2010; de Miranda et al., 2010; Di Prisco et al., 2011); Israeli acute paralysis virus (IAPV) and Deformed wing virus (DWV), most often transmitted by the ectoparasitic *V. destructor* (Cox-Foster et al., 2007; Yue et al., 2007; Blanchard et al., 2008); infectious disease of honeybee larvae 'chalkbrood disease' caused by the fungus *Ascosphaera apis*, affecting the overall health of bee larvae by rapidly decreasing the rate of larval feeding and substantially decreasing the expression of storage proteins, as well as affecting the differential expression of genes associated with stress-related cellular processes and immune responses, leading to gradual deterioration of the colony (Aronstein et al., 2010); honeybees parasitized by the phorid fly *Apocephalus borealis* show symptoms, such as disorientation and loss of equilibrium, could be a new threat to honeybees (Core et al., 2012); the widespread development of genetically modified (GM) crops in which herbicide resistance, pesticide resistance, and insect-killing genes are expressed, which may produce sublethal effects on honeybees (Huang et al., 2004; O'Callaghan et al., 2005; Duan et al., 2008; Eischen and Graham; 2008); electromagnetic radiation from mobile phones may be responsible for affecting the foraging behavior of honeybees (Hsu et al., 2007); nutritional stress produced on honeybees during migratory commercial beekeeping due to their use in the pollination on monocrops therefore they lack a natural defense system (Sharpe and Heyden, 2009; Spivak et al., 2011; Alaux et al., 2010b); transportation stress caused by low temperature brood rearing (Medrzycki et al., 2010); lack of genetic diversity due to mating with a single male degrades the quality of queen bee by producing offsprings of low genetic quality and such colonies provide no colonization resistance to pathogens (Mattila et al., 2012); sensitivity to numerous pesticides, disrupting several honeybee behaviors such as feeding, learning performance, orientation, and navigation (Thompson, 2003; Decourtye et al., 2003; Rortais et al., 2005; Desneux et al., 2007; Girolami et al., 2009; Maini et al., 2010; Henry et al., 2012; Belzunces et al., 2012); and combinational theory in which synergistic interactions among multiple biological, environmental, and chemical factors could derive CCD (Johnson et al., 2009a,b; Ratnieks and Carreck, 2010; Bromenshenk et al., 2010; Mullin et al., 2010; Alaux et al., 2010a; Wu et al., 2011; Spivak et al., 2011; Pettis et al., 2012; Nazzi et al., 2012; Lu et al., 2012).

The existence of high frequency of IAPV in asymptomatic hives in samples from Argentine provinces suggests that IAPV is not a good marker for CCD (Reynaldi et al., 2011). Co-infection with two pathogens (invertebrate iridovirus iridescent virus, IIV; and *N. ceranae*) was more lethal to honeybees than either pathogen alone, suggesting that such association could be critical to CCD (Bromenshenk et al., 2010). However, the validity of this relationship still remains obscure due to lack of evidence for the presence of an Iridovirus in healthy or CCD colonies in the USA and Israel (Tokarz et al., 2011; Foster, 2012). Also all parasites and pathogens could contribute to colony decline, but none seems to be the sole cause of CCD. Mixed fungal infections in honeybee brood, such as chalkbrood caused by the fungus *A. apis* and a pollen fungus *A. atra*, could enhance honeybee mortality compared to single infection (Vojvodic et al., 2012). The theory of short telomeres and premature aging, based on exhausted telomere reserves causing immune suppression leading to the death of foragers, needs further investigation (Stindl and Stindl, 2010). No conclusive data are available on other theories (GM crops, queen bee quality, electromagnetic radiation, and nutritional stress. A variety of unusual ribosomal RNA fragments abundantly found in guts of CCD bees could be linked with picorna-like viral infection, leading to arrested translation (Johnson et al., 2009a). Sublethal doses of miticides (coumaphos,

Table 1
Comparison of symptoms in large-scale colony losses between past and present.

Past Colony Losses	CCD
-Loss of 10–30% colonies	-Loss of 50 to 90%
-Dead bees in or around the hive from bee diseases	-Very few dead bees in or around the hive
	-Sudden disappearance of worker honeybees
	-Queen is healthy and still laying eggs
	-Only a few young bees survive
	-Presence of adequate food and capped brood in colonies
-Infection can be controlled with a specific antibiotic, anti-fungus and other chemicals	-No outward signs of infection, disease, pests, or parasites exist
-Multifactorial syndrome	-Multifactorial syndrome
-Inflammation may be a potential molecular mechanism involved	-ROS-induced oxidative stress may be a potential molecular mechanism involved

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Table 2
A synergistic interaction between neonicotinoid pesticides and other stressors most likely contributes to CCD of pollinators.

Country	Multiple stressors in the colony and/or in bee samples	Potential impact on colony collapse	Refs.
France	-Residues of imidacloprid and its metabolite 6-chloronicotinic acid	None	Chauzat et al. (2009)
France	-Low levels of imidacloprid	None	Bonmatin et al. (2005); Chauzat et al. (2006)
Spain	-Fluvalinate and chlorfenvinphos were present, but no neonicotinoid residues were detected	None	Bernal et al. (2010)
Spain	-Imidacloprid, Israeli Acute Paralysis virus, and fipronil	None	Garrido-Bailón et al. (2010)
Spain	-Low levels of honeybee viruses (DWV, IAPV, SBV, BQCV, KBV)	None	Antúnez et al. (2012a)
Belgium	-Four different bee colonies: three different contaminations with imidacloprid, fenoxycarb, and indoxacarb, and one control hive	Disruption in bee's foraging activity	Beliën et al. (2009)
Belgium	-Amidacloprid-treated corn study (agricultural fields located within a radius of 3,000 m around the hives)	None	Nguyen et al. (2009)
France	-Imidacloprid and *N. ceranae*	Honeybee colony collapse	Alaux et al. (2010a)
Greece	-Imidacloprid and multiple pathogens (imidacloprid; viruses: CBPV, ABPV, DWV, SBV, BQCV; *N. ceranae, V. destructor*) were identified in colonies	Honeybee colony collapse	Bacandritsos et al. (2010)
Italy	-Thiamethoxam, clothianidin, and imidacloprid were present in guttation drops (Feeding)	Honeybee colony Collapse	Girolami et al. (2009); Maini et al. (2010)
Italy	-Direct aerial powdering of dust (clothianidin and imidacloprid-treated seeds) on foragers in free flight near drilling machine and high air humidity	Lethal poisoing by dust and humidity	Girolami et al. (2011); Marzaro et al. (2011)
USA	-Multiple viruses, higher loads of pathogens, and pesticides	Honeybee colony Collapse	Cox-Foster et al. (2007); Johnson et al. (2009); vanEngelsdorp et al. (2009)
USA	-Imidacloprid and *Nosema* spp. (*N. apis* and *N. ceranae*)	Honeybee colony collapse	Pettis et al. (2012)
USA	-Miticides and agrochemicals (98 pesticides and metabolites)	TBD	Mullin et al. (2010)
USA	-Imidacloprid-treated hives (sub-lethal levels added in high-fructose corn syrup, an *in situ* study)	Honeybee colony collapse	Chensheng et al. (2012)
USA	-Developmental exposure to pesticides in brood comb increases susceptibility to *N. ceranae*	Honeybee health decline	Wu et al. (2012)
UK	-Imidacloprid (Trace levels in diet under laboratory and semifield conditions using adult bees and colonies)	None	Cresswell, 2011
UK	-Imidacloprid (Using field-realistic levels)	Bumblebee colony collapse[a]	Whitehorn et al. (2012)
Brazil	Imidacloprid (increasing doses added in the diet given to individual worker bee larvae)	Adult stingless bee colony collapse[b]	Tomé et al. (2012)
South America (Uruguay)	-Pesticides (fluvalinate, flumetrin, amitraz and coumaphos); *N. ceranae; V. destructor;* and viruses (CBPV, BQCV, SBV, and DWB) were identified in colonies	None	Antúnez et al. (2012b)
Uruguay	-Imidacloprid was detected in propolis and honeycombs at higher levels than required for bee disorientation, however, fipronil was detected in honey and dead bee samples at toxic levels	Honeybee colony collapse that may be due to acute toxicity	Pareja et al. (2011)

TBD, To be determined; viruses (CBPV, Chronic bee paralysis virus; DWV, Deformed Wing Virus; IAPV, Israeli Acute Paralysis; SBV, Sacbrood virus; BQCV, Black Queen Cell Virus; KBV, Kashmir bee virus).
[a] Bumblebee, *B. terrestris.*
[b] stingless bee, *M. quadrifasciata*

tau-fluvalinate) simultaneously present in-hive are more toxic to honeybees than used alone support a synergistic action (Johnson et al., 2009b). A combination of selective stressors found in the colony (Table 2) suggests the possibility of a synergistic action among different factors (Mullin et al., 2010; Cox-Foster et al. 2007; Johnson et al., 2009b; vanEngelsdorp et al., 2009; Nazzi et al., 2012; Pettis et al., 2012; Bacandritsos et al., 2010).

Laboratory studies in Europe and USA have demonstrated that small amounts of neonicotinoids (alone or combined with other pesticides or pathogens) cause disorientation, reduced communication, impaired learning and memory, reduced longevity, and disruption of honeybee brood cycles (Medrzycki et al., 2010; Decourtye et al., 2003, 2004a,b; Iwasa et al., 2004). According to several recent studies, sublethal exposure to imidacloprid at the colony level promotes *Nosema* spore production in honeybee's gut (Pettis et al., 2012); feeding of imidacloprid in high fructose corn syrup (HFCF) to honeybees exhibits symptoms consistent to CCD (Lu et al., 2012); field-realistic levels of imidacloprid reduce colony growth and queen production of bumble bee *Bombus terrestris* (Whitehorn et al., 2012); and increased doses of imidacloprid given in diet to stingless bee workers *Melipona quadrifasciata* larvae show negative impact on the development of mushroom bodies and walking behavior (Tomé et al., 2012). Collective findings support the combinational theory of CCD,

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and neonicotinoids could be one of the key contributing factor to CCD.

2.2. Temporal association between pesticides and CCD

Pesticides are prominent part of honeybee environment in the hive as well as in the field. Mixtures of pesticide formulations and types have been found in bees, wax, stored food, and the pollen and nectar on which bees forage. Honeybees bring back these pesticides from directly treated crops and from nearby untreated plants (dandelions). In 1993, Bayer's systemic pesticide imidacloprid (a trade name Gaucho), belonging to a class of neonicotinoids, was introduced in France to treat sunflower and corn seeds to protect against major pests. French beekeepers blamed imidacloprid for "Mad bee disease" for the drastic decline in the honeybee population. Therefore, its use as a seed dressing for sunflower was banned in 1999, and on corn seeds suspended 5 years later. In Germany, imidacloprid was used mainly in the production of rape, sugar beet and corn. Both Germany and France have faced colony losses, resulting in a large fall in honey production and loss in the output of apples, pears and rape due to significant loss in honeybees. The use of neonicotinoids as seed treatment has been banned in Germany, France and Italy till further investigation. Imidacloprid and clothianidin were registered in the USA in 1994 and 2003, respectively. Corn seed companies in the USA started marketing seeds treated with a 5X level of neonicotinoids (1.25 mg/seed vs. 0.25) in 2004. CCD was first experienced in the USA in the fall 2006 (Cox-Foster et al., 2007). Since then the elevated average winter losses have been reported in migratory operations wintering in the USA. Since 2006, CCD has been observed in the Canadian provinces, several European countries, Asia, and South and Central America. However, a bee kill in Germany in 2008 was not CCD but rather an accident. Lethal effect of clothianidin occurred due to improper use of sowing equipment during plantation of corn seed, emission of clothianidin dust into the air was pushed by the wind onto neighboring canola fields. Collective evidence shows a temporal association with CCD. Formamidines belong to a structurally novel group of non-systemic pesticides such as chlordimeform, formetanate, and amitraz. Chlordimeform (Galecron®, Fundal®) registration was voluntarily withdrawn in 1988–1989 in most countries. Since 1969, formetanate HCl (Carzol®) has been registered and used for insect control in crops. Amitraz (Miticur®) was registered in 1992 in the USA with the active ingredient incorporated in a plastic strip and suspended between brood frames. In 1994, amitraz strips were withdrawn from the US market due to bee mortality. Amitraz is now available in the USA as a veterinary miticide (Taktic®), which is not permited in bee colonies. Despite the status of amitraz, it is being used in American apiaries because lower amounts of amitraz metabolites are still being detected in bee-wax and pollen (Mullin et al., 2010; Berry, 2009). The presence of formamidine residues combined with neonicotinoids and other stressors could exacerbate toxic effects to honeybees, contributing to CCD.

2.3. Geographical association with CCD

According to recent survey data gathered by prevention of honeybee Colony LOSSes (COLOSS), a global network consisting of 55 countries, there is a wide variation in overwintering honeybee losses based on geographical regions. Average overwintering honeybee losses vary in different states of the USA, in 6 provinces of Canada, and across Europe. However, a negligible variation of mean honeybee winter losses reported in 5 provinces of China and in south Europe could be due to the absence of factors that cause colony losses (van der Zee et al., 2012). A large variation of colony losses within the country may be due to combination of different factors associated with colony losses (Potts et al., 2010). Instead a large variation in average percent mortality in different countries may be due to differences in survey efforts such as size of the operation, sampling strategy, geography, colony management (migratory or stationary), and survey conductance by beekeepers or regulatory/research officials. Neonicotinoids are applied as seed treatments to crops including sunflower, corn, canola and cotton. Neonicotinoids are systemic insecticides. Therefore, they are translocated to the whole plant (flowers, pollen, and nectar) and can even reach the leaves through guttation when applied to seeds, revealing ways by which bees and other pollinators can be exposed to these compounds (Girolami et al., 2009; Reetz et al., 2011; Tapparo et al., 2011).

In some field studies conducted with neonicotinoids, any effects observed on honeybees do not directly represent the concentration of pesticide used on the crop because observed effect may be reduced as a result of bees foraging to dandelions (Stokstad, 2007; Hopwood et al., 2012). This could be the reason for no toxic effect of pesticides found on the colony (Table 2) in an amidacloprid-treated corn study in Beligium (Nguyen et al., 2009). Depending on the soil conditions in a given geographic area, the half lives of neonicotinoids vary in a range of 148–1155 days, therefore they may persist in the soil for a longer time. Using whole-genome microarray analysis, a considerable geographical variation has been observed in the gut gene expression between samples collected from the USA (east and west coasts) from CCD and control foragers (Johnson et al., 2009a). In west coast bees, less abundance was found in transcripts of genes related to basic cellular processes, involving ribosomal and mitochondrial function. West coast bees seem more severely affected than east coast bees, but no changes were observed in the expression of pesticide and immune response genes. In spite of considerable geographical variation in gene expression, microarray does not explain the cause of CCD.

Low levels of imidacloprid are found in French pollen collected from corn, sunflower and canola (Table 2) with no effect on colony decline (Bonmatin et al., 2005; Chauzat et al., 2006). In 2009, sudden death affecting colony population decline of adult honeybees in Greece reported contamination of some bee samples with imidacloprid (14–39 ng/g tissue) as well as symptomatic workers were positive for *V. destructor*, *N. ceranae* and multiple viruses (Table 2), implicating the synergistic action due to presence of various stressors (Bacandritsos et al., 2010). Honeybees pollinate many crops including almonds, apples, grapes, corn, soybeans, and canola, cotton, and others. Except 0.2% organic corn, all corn seed planted in North America is coated with neonicotinoids (vanEngelsdorp et al., 2009; Mullin et al., 2010; Krupke et al., 2012). Direct aerial powdering of neonicotinoids-treated corn seeds dust from the planting machine on foragers (no contact with vegetation) with high air humidity resulted in high bee mortality in Italy, suggesting the synergistic effect of high dose and humidity (Girolami et al., 2011; Marzaro et al., 2011). A recent US study has reported the presence of neonicotinoids in the soil of treated as well as unplanted fields, implicating deposition on the flowers and uptake by the root system or both (Krupke et al., 2012). Neonicotinoids detected in the corn pollen inside the hive and in dead bees around the hives came either from consumed pollen or by soil/planter dust. In this case bees exerted acute poisoning (not CCD) from treated corn seeds dust being blown during corn seed planting. Geographical association with colony decline should be relevant to all pollinators.

3. Biogenic amines

The biogenic amines include the catecholamines (norepinephrine, NOR; epinephrine, EPI; dopamine, DOP), tyrosine metabolites (octopamine, OCT; tyramine, TYR), indoleamine (serotonin, 5-HT),

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Table 3
Classes of biogenic amines-based pesticides, their neuronal targets and actions.

Class	Insensitivity to degradation	Recptor activation	Action	Refs.
Formamidines	-Formamidines inhibit *N*-acetylation reaction by inhibiting *N*-ACT enzyme activity therefore reduce conversion of OCT into *N*-acetyl-p-OCT	Persistent activation of OCTR	Impairment in olfactory learning	Dudai, (1982); Dudai et al. (1986, 1987); Downer et al. (1985), Evans and Gee (1980); Martin and Downer (1989)
	- Formamidines accumulate OCT by inhibiting MAO	Persistent activation of OCTR	Impairment of olfactory learning	Schuntner and Thompson (1978)
Neonicotinoids	-Neonicotinoids are insensitive to AChE	Persistent activation of nAChR	Impairment of learning performance and decrease in olfactory memory	Tomizawa and Casida (2005); Matsuda et al. (2001); Cassida and Quistad (2004); Decourtye et al. (2003), (2004b); Jeschke and Nauen (2008); Aliouane et al. (2009); Han et al. (2010)

OCT, octopamine; OCTR, octopamine receptor; ACh, acetylcholine; nAChR, nicotinic acetylcholine receptor; AChE, acetylcholine esterase; *N*-acetyl-*p*-OCT, *N*-acetyl-*p*-octopamine; *N*-ACT, *N*- acetyltransferase; MAO, monoamine oxidase.

imidazoleamine (histamine, HIS), and an ester of acetic acid and choline (acetylcholine, ACh). Some biogenic amines are preferentially synthesized (such as NOR and EPI in vertebrates, and OCT and TYR in invertebrates); whereas DOP, 5-HT, HIS, and ACh are synthesized in both vertebrates and invertebrates. Major biogenic amines are derived from three different amino acids by single to multistep enzymatic reactions: NOR, EPI, DOP, TYR, and OCT from aromatic amino acid L-tyrosine, 5-HT from tryptophan, and HIS from histidine (Blenau and Baumann, 2001). However, ACh is synthesized from choline and acetyl-CoA. Biogenic amines are stored in synaptic vesicles. Upon their release, they activate corresponding postsynaptic G-protein coupled receptors (GPCRs) and regulate the activity of intracellular secondary effectors, resulting changes in intracellular concentrations of different second messengers responsible for specific cellular responses (Farooqui, 2012a; Park and Adams, 2005; Blenau and Baumann, 2001). Biogenic amines act as neurotransmitter, neuromodulator, and neurohormone in the nervous system of insects (Roeder, 1994, 2005; Roeder et al., 2003; Blenau and Baumann, 2001; Downer, 1990; Evans, 1980). By modifying the synaptic output of relevant neurons, they play crucial roles in the modulation of insect behaviors. Biogenic amines are involved in the regulation of variety of functions such as endocrine and exocrine secretion (Rietdorf et al., 2005; Just and Walz, 1996; Marg et al., 2004); the generation of motor patterns (Claassen and Kammer, 1986); aggression (Stevenson et al., 2005; Robinson et al., 1999); foraging behavior (Schulz and Robinson, 2001; Schulz et al., 2002; Barron et al., 2007); muscle contraction (Bicker and Menzel, 1989); and different forms of learning and memory formation (Farooqui, 2012b, 2007a,b; Farooqui et al., 2003; Schwaerzel et al., 2003; Johnson et al., 2011; Gauthier, 2010; Dacher and Gauthier, 2008; Hammer, 1997; Meller and Davis, 1996). Therefore, any disruption in biogenic amines-mediated signaling may impair complex behaviors including olfactory learning and memory.

4. Pesticides: A possible contributing factor to CCD

In agriculture, pesticides have been widely used throughout the world to kill or retard the growth of insects, weeds, fungus and other pests that can endanger food crops and carry diseases. Based on the type and dose, pesticides may be less toxic to target pests, more toxic to non-target plants and animal species, as well as beneficial insects such as honeybees. Pesticides are classified based upon target organism, chemical structure, physical state, and mode of action. Despite the availability of so many theories for CCD mentioned above, I focus on the 'Pesticide Theory' as a key contributing factor to CCD in honeybees because of the following reasons: (1) most colony losses from 1966 to 1979 have been attributable to pesticide exposure (Atkins, 1992), (2) worker honeybees (older) show higher sensitivity to pesticide exposure than younger honeybees that remain inside the hive (Wahl and Ulm, 1983; Rortais et al., 2005), (3) the honeybee genome is markedly deficient in the number of genes encoding detoxification enzymes (such as cytochrome P450 monooxygenases, P450s; glutathione-*S*-transferases; and carboxylesterases) that makes honeybees more sensitive to pesticides (Claudianos et al., 2006), (4) systemic pesticides are absorbed by the plant and translocated to its floral resources such as pollen (Laurent and Rathahao, 2003; Bonmatin et al., 2003, 2005; Bernal et al., 2010), and nectar (Krischik et al., 2007; Bacandritsos et al., 2010), and depending on their concentration they could either alter behavior or kill pollinators, and (5) honeybees exposed to sublethal levels of pesticides exert weakness, significant reduction in taste sensitivity, problems with flying and navigation, impaired olfactory learning, which could lead to abnormal foraging performance (Yang et al., 2008; Decourtye and Devillers, 2010; Han et al., 2010; Decouriye et al., 2003, 2004a,b; Pareja et al., 2011; Henry et al., 2012; Schneider et al., 2012; Teeters et al., 2012). All these observations support 'Pesticide Theory' that chronic or sublethal intoxication could have contributed to reported colony losses.

4.1. Biogenic amines-based pesticides

Biogenic amines-based pesticides classes discussed here include (1) nicotine-based pesticides (neonicotinoids) that mimic ACh action by acting on insect nicotinic acetylcholine receptors (nAChRs), and (2) OCT-based pesticides (formamidines) that mimic OCT action by acting on octopaminergic receptors (OCTRs). Following section discusses two classes of biogenic amines-based pesticides (Table 3) in relation to the molecular basis for their action in insect neurotransmission.

4.1.1. Neonicotinoids

Neonicotinoids are systemic nicotine-based pesticides that move inside a plant following absorption by the plant and are widely used on many pollinated agricultural crops. This group of pesticides include nitro-substituted (clothiandin, dinotefuran, imidacloprid, thiamethoxam, and nitenpyram) and cyano-substituted (acetamiprid and thiacloprid) compounds (Fig. 1). Neonicotinoids mimic the action of ACh by selectively binding to postsynaptic nAChRs as agonists in the insect brain (Matsuda et al., 2001), therefore often referred as ACh mimics. The nAChRs belong to the cys-loop ligand-gated ion channel superfamily (Karlin, 2002). The synaptic activation by ACh is terminated by AChE enzyme, which rapidly hydrolyzes the ester linkage in ACh. However, nicotine and neonicotinoids are insensitive to action of AChE (Table 3), therefore, neonicotinoids accumulate ACh, resulting in persistent activation of nAChRs (Cassida and Quistad, 2004; Jeschke and Nauen, 2008; Tomizawa and Casida, 2005; Matsuda et al., 2001). Agonist recognition by nAChR involves an interaction with the nitro or cyano sub-

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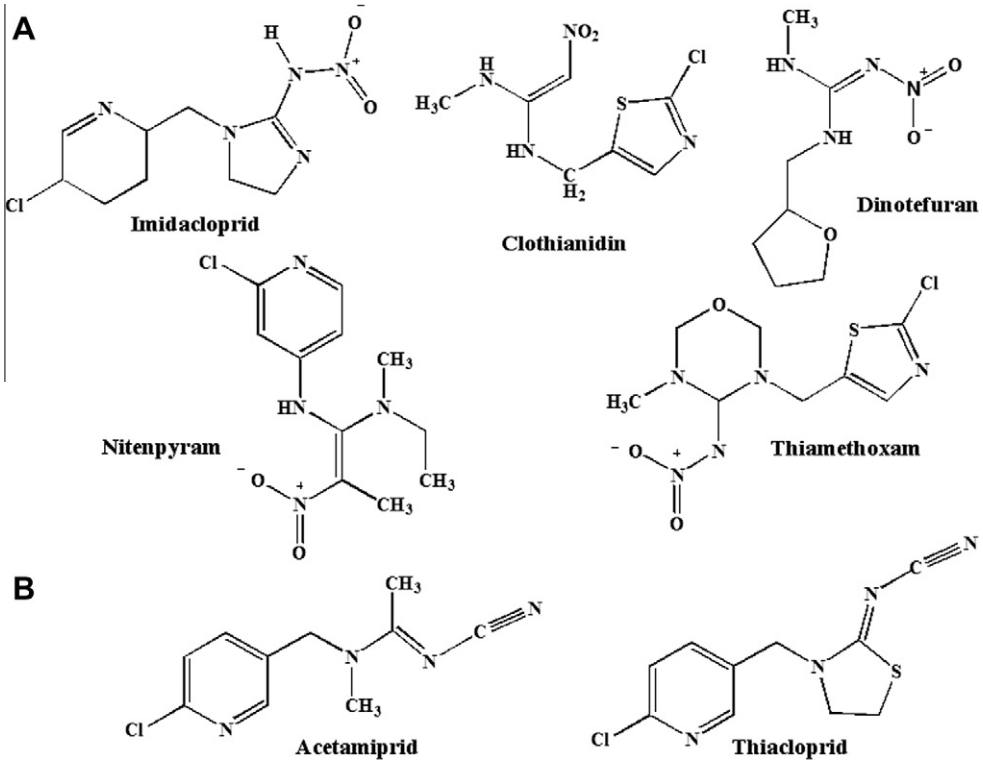

Fig. 1. Chemical structures of main neonicotinoids cited in this article: (A) nitro-substituted neonicotinoids including imidacloprid, clothianidin, dinotefuran, nitenpyram, and thiamethoxam, and (B) cyano-substituted neonicotinoids including acetamiprid, and thiacloprid.

stituent of neonicotinoids in insects, whereas it involves cation-pi interaction with neonicotinoids in mammals and vertebrates (Tomizawa and Casida, 2003, 2005). Neonicotinoids interfere with the transmission of neural messages in insects much more efficiently than in mammals and vertebrates, suggesting that a nitro or a cyano group of neonicotinoids contributes to their selectivity in insects (Tomizawa and Casida, 2003, 2005; Decourtye and Devillers, 2010). In the laboratory study, topical application of pesticides on honeybees, has reported LD50 values of nitro-substituted neonicotinoids (18 ng/bee for imidacloprid, 22 ng/bee for clothianidin, 30 ng/bee for thiamethoxam, 75 ng/bee for dinotefuran and 138 ng/bee for nitenpyram) and cyano-substituted neonicotinoids (7.1 μg/bee for acetamiprid and 14.6 μg/bee for thiacloprid), suggesting that the nitro-substituted neonicotinoids are more toxic to honeybees than cyano-substituted neonicotinoids (Iwasa et al., 2004). Addition of fungicide (triflumizole) increases honey bee toxicity of acetamiprid to 244 fold and thiacloprid to 1141 fold, respectively, suggesting the possibility of synergistic action (Iwasa et al., 2004). Neonicotinoids seem to be more toxic to honeybees by oral consumption than by contact (Suchail et al., 2000). It should be noted that some neonicotinoids metabolites (such as olefin, nitrosamine, and 5-hydroxyimidacloprid) are more toxic than the parent compound (Suchail et al., 2001, 2004; Nauen et al., 1998, 2001). Another metabolite clothianidin (precursor thiamethoxam) is as toxic as parent compound, and has high affinity to nAChRs (Nauen et al., 2003; Benzidane et al., 2010). These studies suggest that neonicotinoids metabolites act agonistically on nAChRs. It may be possible that most toxic metabolites are produced via oral consumption than by contact in honeybees.

The genome sequencing has revealed diversity in number of nAChRs subunits in different insect species, varying in the range of 10–12 (Sattelle et al., 2005; Jones et al., 2005, 2006; Jones and Sattelle, 2007; Shao et al., 2007). Some important amino acid residues in insect nAChR α and β subunit contribute to neonicotinoid insecticides selectivity, and mutations in selective amino acid residues of these subunits to other residues decrease neonicotinoid insecticides affinity, resulting in neonicotinoid insecticide resistance (Liu et al., 2008). Thus, amino acid residues in insect α and β subunits of nAChRs determine neonicotinoid's selectivity, implicating that the subunit composition of nAChRs defines the molecular basis for their broad functional diversity.

4.1.2. Amidine compounds

Formamidines, a group of acaricidal compounds, are chemically characterized by the presence of a phenylamidine moiety (Hollingworth, 1976; Hollingworth and Murdock, 1980). This class of insecticides includes commercially marketed compounds including amitraz (*N′*-(2,4-dimethylphenyl)-*N′*-[(2,4-dimethylphenyl)-imino]methyl-*N*-methylinethanimidamide); amitraz meta bolite (*N′*-(2,4-dimethylphenyl)-*N*-methylformamidine, BTS-272 7l); chlordimeform (*N′*(4-chloro-*O*-tolyl)-*N* dimethyl formamidine, CDM); its degradable product *N*-demethylchlordimeform, DCDM); and formetanate (*N*,*N*-dimethyl-*N′*-[2-methyl-4-[[(methylamino) carbonyl]oxy]phenyl]methanimidamide) (Fig. 2). Formamidines are octopaminergic agonists that mimic the action of octopamine (OCT; 1-(*p*-hydroxyphenyl)-2-aminoethanol) in elevating adenylyl cyclase (AC) activity in intact nerve cords of *Periplaneta Americana* (Nathanson and Hunnicutt, 1981). Increased cyclic adenosine monophosphate (cAMP) levels in brain and peripheral tissues cause tremors and convulsions. These effects are specific to OCT rather than dopamine or serotonin (Downer et al., 1985). Formamidines have also been reported to mimic the actions of OCT in the light organ of firefly, and at the neuromuscular junction of locust (Hollingworth and Murdock, 1980; Evans and Gee, 1980). At lower doses, formamidines suppress pest mating, reproduction, and feeding behavior (Knowles, 1982; O'Brien et al., 1985). Both DCDM and OCT increase the phosphorylation of proteins that are also phosphorylated by exogenous cAMP-dependent protein kinase in

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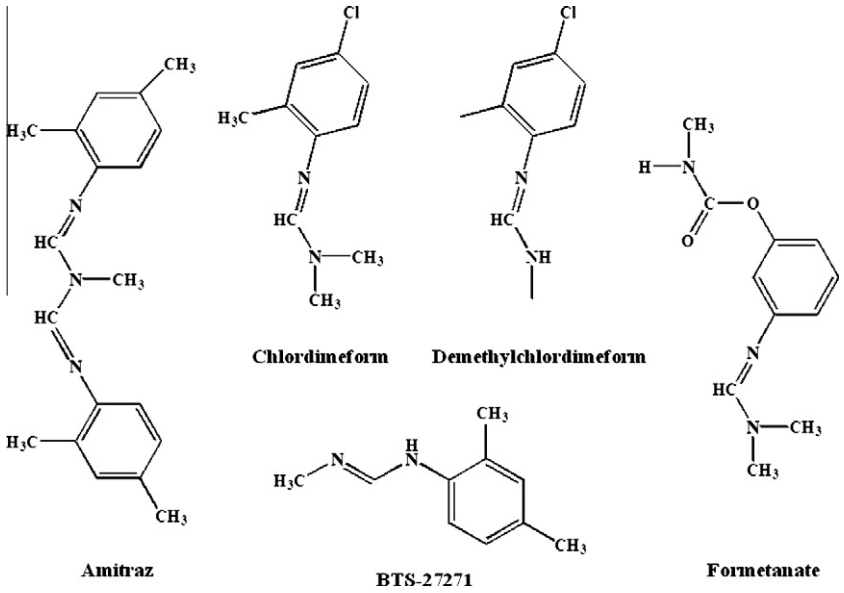

Fig. 2. Chemical structures of main formamidines cited in this article: amitraz and its metabolite BTS-2727 l, chlordimeform and its metabolite *N*-demethylchlordimeform; and formetanate.

the two-spotted spider mite, confirming the agonistic effects of formamidines through OCT-sensitive AC (Ismail and Matsumura, 1989). Formamidines have been shown to induce motor excitation and impair olfactory learning in *Drosophila* (Dudai et al., 1986).

Amitraz influences honeybee behavior, therefore it has been used to control several agricultural pests, including parasitic mites in honeybees (Liac et al., 2004; Elzen et al., 2001; Floris et al., 2001). The exposure to amitraz solution causes acute toxicity in honeybee larvae by increasing apoptotic cell death in the midgut (Gregorc and Bowen, 2000). Both CDM and DCDM have similar affinity for displacing [^{3}H]OCT from its specific membrane-associated binding sites in *Drosophila*, while amitraz is considerably more potent (Dudai, 1982; Dudai et al., 1987). Formamidines inhibit *N*-acetyltransferase (*N*-ACT) activity, therefore inhibit conversion of OCT into *N*-acetyl-*p*-OCT in insect tissues (Table 3). DCDM inhibits *N*-acetyl-*p*-OCT production in Malpighian tubule preparations as well as in intact tissues, whereas amitraz and BTS-27271 prevent *N*-acetyl-*p*-OCT generation only in intact tissues of *P. americana* (Martin and Downer, 1989). Formamidines exert their effect in the CNS of target species by interacting and over-stimulating octopaminergic receptors (Evans and Gee, 1980; Dudai et al., 1986). Furthermore, formamidines inhibit monoamine oxidase (MAO) activity (Table 3), therefore prevent biogenic amines degradation, accumulate OCT, and alter the behavioral plasticity in insects (Atkinson et al., 1974; Schuntner and Thompson, 1978; Dudai et al., 1987). On the other hand, formetanate is a bifunctional pesticide with formamidine and carbamate groups. Its current value lies in the control of organophosphate and carbamate-resistant pests. Formetanate is expected to be non-persistent in soil and aquatic environments due to rapid photolytic and hydrolytic transformation. It inhibits acetylcholinesterase (AChE) by reversible carbamylation of the serine hydroxyl group of the enzyme (Knowles and Ahmad, 1971), causing short term cholinergic poisoning. Like other formamidines, formetanate inhibits MAO that results in the accumulation of OCT. It exerts very low toxicity to bees when exposed to residues on plants, but it is moderately toxic when applied directly to foragers in the field or colonies.

Collectively, chronic exposure of biogenic amines-based pesticides to honeybees disrupt their neural functions including learning performances.

5. Olfactory learning and memory in honeybees

The neural bases for olfactory learning are distributed across several areas such as antennal lobes (ALs) and mushroom bodies (MBs) in the honeybee brain. This suggests the involvement of different areas to perform different phases of memory, such as learning (acquisition), short term and long term memory (consolidation), recall, and discrimination among odors (Farooqui, 2007a,b). Olfactory memory plays an important role in many aspects of honeybee behavior, including recognition of nestmates, foraging, food preferences, hive location, and navigation (Menzel et al., 2005; Menzel and Müller, 1996; Giurfa, 2003, 2007). Navigation of honeybees requires learning of the directions and distances of their travels between nest and food sources. Honeybees relate all navigational decisions to the origin of their flight path, and apply a navigational motor routine to bring them back to their hives by recognizing the landmark and multiple associations between landmarks and respective return heading and distance to their hives, suggesting that honeybees navigate according to a map-like organization of spatial memory (Menzel et al., 2005). Honeybees have sophisticated sensory systems, including well developed learning and memory capacities, therefore serve as a valid model to study the underlying mechanism of learning and memory. Any disruption in olfactory learning and memory may result in leaving a negative impact on their foraging performance, leading to CCD. Assuming that bigenic amines-based pesticides are indeed one of the key contributing factors to CCD, then following sections will help understanding the association between neurochemical systems and olfactory learning and memory in honeybees.

5.1. Cholinergic system: Association with olfactory learning and memory

ACh is the most abundant excitatory neurotransmitter in the insect brain. There are two types of ACh receptors: (1) nicotinic ACh receptors (nAChRs) and muscarinic ACh receptors (mAChRs). Both receptor subtypes are activated by ACh, but most abundant ones are nAChRs. The nAChRs are ionotropic receptors - ligand-gated

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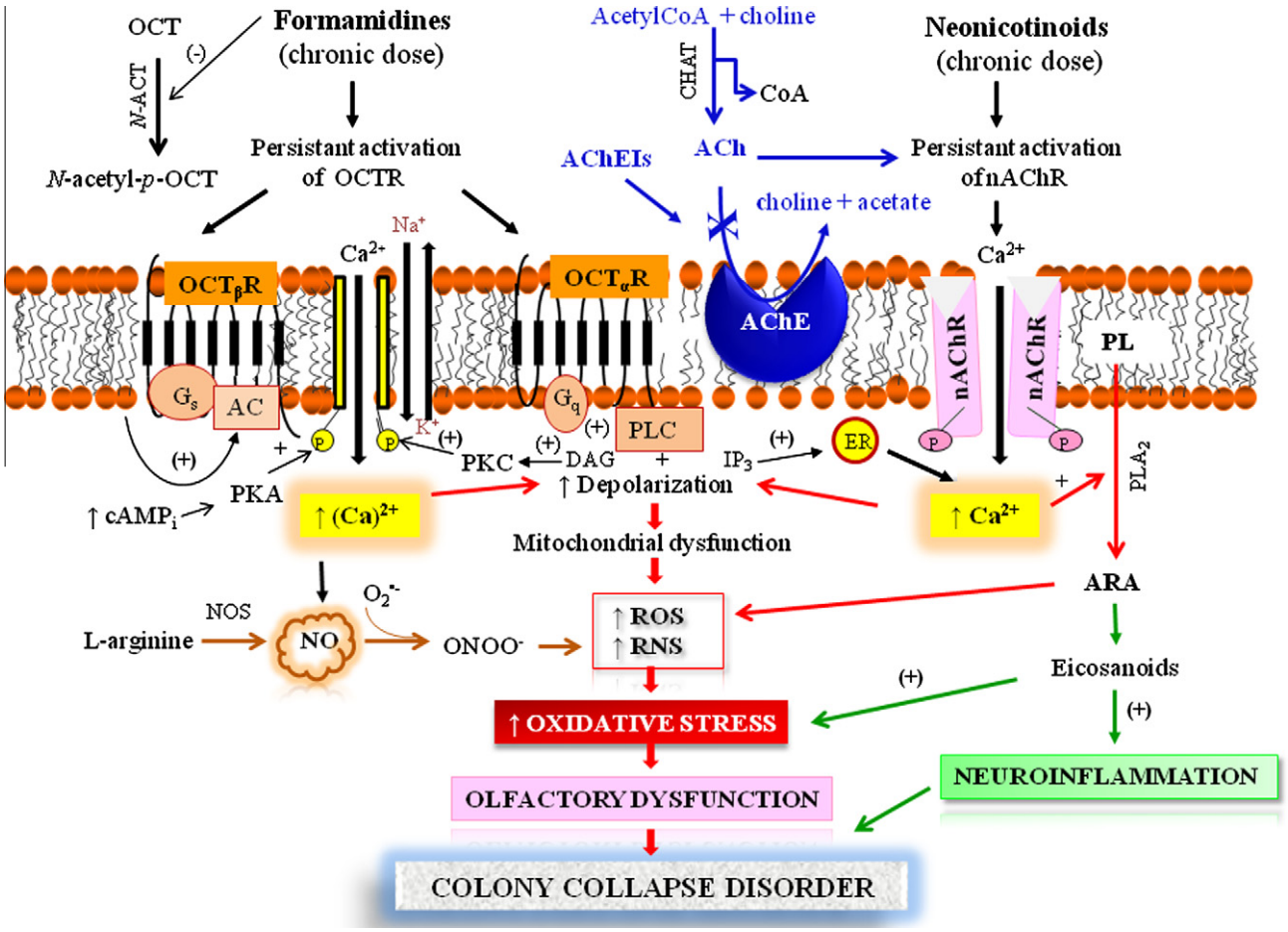

Fig. 3. Hypothetical scheme showing a link among biogenic amines-based pesticides, learning and memory, and CCD. $[Ca^{2+}]_i$, intracellular calcium; ROS, reactive oxygen species; RNS, reactive nitrogen species; NOS, nitric oxide synthase; NO, nitric oxide; PL, phospholipids; PLC, phospholipase C; IP_3, 1,4,5-trisphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; ARA, arachidonic acid; ER, endoplasmic reticulum; PKC, protein kinase C; PKA, protein kinase A; AC, adenylyl cyclase; CHAT, choline acetyltransferase; AChE, acetylcholine esterase; AChEIs, acetylcholine esterase inhibitors; OCT, otopamine; OCT_α-R, octopamine α receptor; OCT_β-R, octopamine β receptor; *N*-ACT, *N*-acetyltransferase (*N*-ACT); CCD, colony collapse disorder.

ion channels. They mediate fast cholinergic synaptic transmission in insect as well as vertebrate nervous systems. The nAChRs are activated by nicotine. The influx of cations causes depolarization of the plasma membrane, resulting in rapid post-synaptic depolarization in neurons that affects the release of neurotransmitters (Lee and O'Dowd, 1999). In contrast, the mAChRs are metabotropic, G-protein coupled receptors, which are more sensitive to muscarine than nicotine. Muscarinic-like receptors in honeybees are involved with information retrieval but not in acquisition or consolidation processes (Gauthier et al., 1994), suggesting that mAChRs deal with specific stage of memory formation.

Due to great abundance within the insect brain, nAChRs are the targets for insecticide development. The nAChRs present in the antennal lobes of the honeybee brain differ from the ones found in the mushroom bodies in their pharmacological profile and ionic permeability, suggesting that differential expression of these receptors may perform different functions (Barbara et al., 2008). The ACh-elicited currents in the antennal lobe elicited by nicotinic agonists (nicotine and imidacloprid) can be blocked by nicotinic antagonists (methyllycaconitine, dihydroxy-β-erythroidine and α-bungarotoxin), supporting the presence of nAChRs (Barbara et al., 2008). In the honeybee antennal lobe, the excitatory cholinergic transmitter system is operated by nAChRs and two inhibitory networks, represented by GABA and glutamate receptors (Grünewald et al., 2004; Barbara et al., 2005). Four nAChR subunits (Apisα2, Apisα3, Apisα7–1, and Apisα7–2), identified in the honeybee *A. mellifera* brain regions are responsible for functions, such as learning and memory, visual processing, and olfactory processing (Jones et al., 2006; Thany et al., 2003). Nicotine injections in the honeybee brain show improvement in subject's short term memory (Thany and Gauthier, 2005). Mecamylamine (nAChR antagonist) in the honeybee brain inhibits olfactory learning or recall, depending upon the site of injection in the brain (Lozano et al., 1996, 2001). Btx-sensitive nAChRs participation in the long-term memory and Btx-insensitive nAChR participation in recall suggest that nAChRs are critical for learning and memory (Dacher et al., 2005). Imidacloprid is highly toxic to *A. mellifera* (Suchail et al., 2004). At sublethal doses, imidacloprid has been reported to alter foraging and learning in honeybees (Guez et al., 2001; Lambin et al., 2001; Decourtye et al., 2004a,b). Laboratory bioassays conducted with subchronic exposure of honeybees to sublethal doses of neonicotinoids using olfactory conditioning paradigm suggest that after exposure to some of these compounds, honeybees either fail to discriminate between known and unknown odorants or significantly impair their learning and memory performance (Aliouane et al., 2009).

Feeding honeybees sugar solution containing imidacloprid or deltamethrin (synthetic pyrethroid) decreases food foraging activity and slows down the activity at the hive entrance (Decourtye et al., 2004b). Imidacloprid shows negative effects on olfactory learnt discrimination task in both semi-field and under laboratory

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conditions while deltamethrin shows no such impact on associative learning ability, suggesting the selective role of neonicotinoids at sublethal doses on the impairment of olfactory learning and memory (Decourtye et al., 2004b). Similarly, sublethal doses of thiamethoxam (chronically administered orally or by contact) to honeybees either significantly decrease olfactory memory (24 h after learning at 0.1 ng/bee) or significantly impair learning performance with no effect on memory at (1 ng/bee). Moreover, the developmental exposure to pesticides in brood comb increases the susceptibility of honeybees to *N. ceranae* infection (Wu et al., 2012), implicating that exposure of *N. ceranae* and pesticide contributes to honey bee health decline. Thus, exposure of neonicotinoids to honeybees in agricultural field or in the hive may be either highly toxic or moderately toxic depending on the chemical nature of the compound, the route and the mode of exposure (acute/ subchronic/ or chronic), synergistic action if exposure deals with more than one compounds as well as presence of other external factors.

5.2. Aminergic system: association with olfactory learning and memory

OCT carries out a prominent role in modulating rhythmic as well as complex behaviors, including learning and memory in insects. The restrained honeybees can learn to associate an odorant with a sucrose reward. OCT injected into the antennal lobe of the honeybee brain provides reinforcing properties (an odor can be paired with injections of OCT as a substitute for sucrose), suggesting that OCT plays a role in inducing associative olfactory learning in honeybees. However, OCT injected into the mushroom body calyces of the honeybee brain leads to consolidation after conditioning, but does not lead to acquisition (Hammer and Menzel, 1998). Injections of OCTR antagonist (mianserin) or OCTR double-stranded RNA in the antennal lobes of the honeybee brain inhibit olfactory learning and recall, supporting OCT neuromodulatory role in olfactory processing (Farooqui et al., 2003). Application of OCT in defined areas of brain increases sensitivity to olfactory stimuli and learning behavior (Page et al., 2006). OCT is required for the foraging behavior of insects (Schulz, et al., 2001, 2002). However, OCT may not be the reinforcement mediating transmitter for all forms of learning in honeybees. Post-injections of OCT in the antennal lobes of the honeybee brain result in a rapid increase of protein kinase A (PKA) activity (Hildebrandt and Müller, 1995a) in the same manner as in chemosensory stimulation (sucrose) (Hildebrandt and Müller, 1995b), supporting the view that OCT plays a neuromodulatory role. Long-lasting activation of protein kinase C (PKC) in the antennal lobes of honeybee induces olfactory memory (Grünbaum and Müller, 1998). OCT modulates AC and phospholipase C (PLC) pathways, which are linked with the activation of PKA and PKC and generation of cAMP as well as diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (Ins-1,4,5-P_3, IP_3), respectively. Induction of iron-mediated oxidative stress in the antennal lobes of the honeybee brain causes olfactory dysfunction, which can be reversed by iron chelator or glutathione. However, mianserin or OCTR-dsRNA-mediated inhibition of olfactory learning and memory can not be reversed by iron chelator or glutathione (Farooqui, 2008), implicating that the formation of ROS in the honeybee brain may be another cause of olfactory dysfunction.

Formamidines are structurally related to OCT and interact with the OCTRs [($OCT_{\beta}R$ via stimulatory G protein (Gs) and $OCT_{\alpha}R$ via another stimulatory G protein (Gq)] in the central nervous system. Formamidines inhibit *N*-ACT that prevents conversion of OCT into *N*- acetyl-*p*-OCT, resulting in persistent activation of OCT receptors, which is rather toxic to insects. Formamidines stimulate AC enzyme activity that leads to increased cAMP levels in the central nervous system (CNS) and peripheral nervous system (PNS) (Downer et al., 1985; Hollingworth and Murdock, 1980; Evans and Gee, 1980). Upon feeding, formamidines impair olfactory learning that disturbes recognition of an olfactory cue in *Drosophila melanogaster* (Dudai et al., 1986, 1987). At lower doses, formamidines decrease pest mating, reproduction, feeding, and alter other behaviors (Knowles, 1982; O'Brien et al., 1985). Amitraz activates AC but less than 50% of OCT response, suggesting that amitraz acts as a partial agonist on OCTR (Dudai et al., 1987). Amitraz-mediated activation of AC can be blocked by phentolamine and to a lesser degree by propranolol, resembling OCT effect.

To date, only OCTR that has been cloned and characterized from the honeybee brain is refered as AmOA1 (Grohmann et al., 2003), which is an ortholog of an OCTR preferentially expressed in mushroom bodies (OAMB) of *Drosophila* (Han et al., 1998). We have previously reported the occurrence of this receptor in the antennal lobes as well as in the rest of the brain (brain homogenate excluding antennal lobes) of *A. mellifera* by western blotting using an antiserum raised against a peptide selected from an intracellular region (IL4) of AmOA1 sequence. The expression of four possible OCTRs (78 and 72 kDa in the antennal lobe homogenate, but 78, 72, 60, and 48 kDa proteins in the brain homogenate) suggests that OCTRs are differentially expressed in the honeybee brain. The intensity of these protein bands decreases with OCTR dsRNA treatment, suggesting that these proteins bands may be different subtypes, different splice variants, and/or different post-translational covalent modifications of OCTR (Farooqui et al., 2004). Further characterization is necessary to determine the molecular nature of these proteins to understand their specific roles underlying olfactory learning and memory processes. OCTRs in the honeybee brain are expressed on GABAergic neurons, located in several regions throughout the brain (the mushroom bodies, the antennal lobes and the central complex), implicating that ligand-binding to OCTRs in the antennal lobe and mushroom body participates in the modulation of inhibitory neurons (Sinakevitch et al., 2011). Thus, modulatory effects of formamidines on olfactory traits (acquisition, memory formation, and retrieval) would depend on the dose of the pesticide as well as on the receptor subtype in the honeybee brain.

6. Chemistry of ROS, mitochondrial energy metabolism, and oxidative stress

Reactive oxygen species (ROS) is a collective term that includes both oxygen radicals such as superoxide ($O_2^{\cdot -}$), hydroxyl ($OH^{\cdot}$), peroxyl ($RO_2^{\cdot}$) and hydroperoxyl ($HO_2^{\cdot}$) radicals and non-radical oxidizing agents such as hydrogen peroxide (H_2O_2), hypochlorite ion (OCl^-) and ozone (O_3) that can be converted into radicals (Farooqui and Farooqui, 2009). ROS are mainly produced by mitochondrial electron transport chain in the reduction of molecular oxygen (O_2) to water, oxidation of polyunsaturated fatty acid (PUFA), and reaction between nitric oxide and $O_2^{\cdot -}$ forming peroxynitrite ($ONOO^-$) that forms additional free radicals (Miquel, 1992). $ONOO^-$ not only interacts with proteins and DNA but also reduces mitochondrial respiration, inhibits membrane pumps, and depletes cellular glutathione levels (Beckman et al., 1992). Other sources of ROS include xanthine/xanthine oxidase, myeloperoxidase, and NADPH oxidase (Farooqui and Farooqui, 2009).

The mitochondrial energy metabolism depends on oxidative phosphorylation by which the oxidoreduction energy of mitochondrial electron transport is converted to synthesize adenosine-5'-triphosphate (ATP). The generation of energy is regulated by electron transport chain complexes located in the inner mitochondrial membrane where O_2 serves as the final electron acceptor for cytochrome-c oxidase (complex IV), which catalyzes the four-electron reduction of O_2 to H_2O. Partially reduced and highly reactive metabolites of O_2 ($O_2^{\cdot -}$ and H_2O_2) are referred as ROS, which are produced by one- and two-electron reductions of O_2, respectively. Superoxide

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dismutase (SOD) dismutates $O_2^{\cdot -}$ to the stable H_2O2, and in turn catalse converts H_2O_2 into water and oxygen (Farooqui and Farooqui, 2009). Unlike $O_2^{\cdot -}$, H_2O_2 is not a free radical but serves as an effective non-radical oxidizing agent for many biological molecules. H_2O_2 reduction in the presence of transition metals (Fe^{2+} or Cu^{2+}) yields one of the most toxic hydroxyl radical ($OH^{\cdot}$) by Fenton reaction. Both $O_2^{\cdot -}$ and $OH^{\cdot}$ are continuously generated during oxidative metabolism in biological systems. The overloading of ferrous iron (Fe^{2+}) potentiates ROS production, which is potentially deleterious in biological systems (Liu et al., 2003). $OH^{\cdot}$ attacks PUFA in membrane phospholipids, abstracts a hydrogen atom to initiate lipid peroxidation and forms a lipid hydrperoxy radical ($RO_2^{\cdot}$) in the presence of O_2. Propogation occurs as $RO_2^{\cdot}$ reacts with an adjacent lipid, abstracts a hydrogen, forms a stable lipid hydroperoxide, and generates another lipid radical to continue the chain reaction for lipid peroxidation (Emerit et al., 2001). Lipid peroxidation is terminated by any chain breaking antioxidant that can intercept the electron from the $RO_2^{\cdot}$ and form a stable compound. Increased Fe^{2+} directly initiates additional lipid peroxidation with accumulated lipid hydroperoxides, producing changes in the membrane structure and functional damage (Kehrer, 2000; Liochev, 1999). ROS are capable of promoting the oxidation of proteins that is initiated by reacting with $OH^{\cdot}$. The course of the oxidation process depends on the availability of O_2 and $O_2^{\cdot -}$ or its protonated form, $HO^{\cdot}_2$ (Stadtman and Berlett, 1997). Oxidative attack on the protein cysteine thiol groups (-SH) results in the formation of oxidized to thiyl radicals (–$S^{\cdot}$), disulfides (–SS–), and sulfenic (–SOH), sulfinic (SO_2H), or sulfonic (–SO_3H) acid derivatives, whereas attack on side-chains of amino acid residues (lysine, arginine, proline, or threonine) forms carbonyl groups (Berlett and Stadtman, 1997; Nyström, 2005; Dalle-Donne et al., 2006). ROS-mediated oxidation of protein leads to protein–protein cross linking, oxidation of amino acid residues side chains, and protein fragmentation. ROS interaction with DNA produces a variety of oxidized base lesions in DNA, including base damage, sites of base loss, and single-strand breaks that may contain modified 3'-ends and apurinic/apyrimidinic sites, and modifications in sugar moiety of DNA, triggering genomic instability (Devasagayam et al., 1993; Budworth and Dianov, 2003; Cooke et al., 2003). A major oxidation product 8-oxoguanine (Cheng et al., 1992). ROS and RNS interact with DNA and cause DNA–DNA and DNA–protein crosslinks and sister chromatid exchanges, increasing functional alterations in the cell (De Bont and van Larebeke, 2004).

Oxidative stress is defined as a condition that is produced due to an imbalance in the cellular prooxidant/antioxidant ratio (Sies, 1985; Halliwell, 2006). Brain is highly susceptible to oxidative stress, therefore, high rate of oxygen consumption, high lipid content, and the relative scarcity in antioxidant enzymes compared to other tissues may account for its vulnerability (Leutner et al., 2001). The imbalance between ROS production and cellular defense mechanism has been reported to markedly increase protein oxidation (Smith et al., 1991; Hensley et al., 1995), lipid peroxidation (Balazs and Leon, 1994; Sayre et al., 1997), and DNA/RNA oxidation (Mecocci et al., 1994; Shan et al., 2003), implicating to neural damage. Excessive ROS from mitochondria and other cellular sources such as nonenzymic oxidation of arachidonic acid (ARA) as well as ROS-activated nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) may potentially destroy a living cell (Farooqui and Farooqui, 2011a,b).

7. Link among pesticides, ROS-mediated olfactory learning and memory and CCD

Pesticides are known to stimulate free radical production, induce lipid peroxidation, and disturb redox state of the cell (Abdollahi et al., 2004). One group of pesticides has been shown to alter receptor function and to modify the energetic status leading to apoptotic and toxic effects, while other group initiates cytotoxicity by permeating the plasma membrane and causing structural perturbation in the membrane bilayer (Mech et al., 2009). Biogenic amines are important mediators and/or regulators of neuronal signaling in the insect CNS and PNS.

ROS-mediated oxidative stress can be induced by environmental stress, aging, and age-related diseases. Loss of olfactory function and oxidative damage to olfactory tissue are early 'pre-clinical' sign of several neurological disorders (Perry et al., 2003; Nelson et al., 2009; Barrios et al., 2007; Doty, 2012; Moberg et al., 2006). The basis for olfactory deficit in these diseases are likely ROS-mediated damage to cholinergic, serotonergic, dopaminergic, and noradrenergic systems. ROS, RNS, transition metal ions (iron, copper and zinc), glycating agents and reactive aldehydes, protein cross-linking and proteolytic dysfunction cause cellular stress that contributes to neurodegeneration and olfactory dysfunction (Babizhayev et al., 2011). A significant olfactory dysfunction has been reported in caged aged honeybee workers versus young workers supports that ROS formation induces with aging (Farooqui, 2007b). Application of ferrous ammonium citrate into the antennal lobes of the honeybee brain inhibited olfactory learning and memory that was reversed by reduced glutathione, implicating that normal cellular redox is crucial for olfactory processing (Farooqui, 2008, 2011). Glutathione *S*-transferases are mainly involved in detoxification of endogenous and xenobiotic compounds and offer oxidative stress resistance in insects. A sigma class glutathione *S*-transferase gene has been recently characterized in the honeybee (*Apis cerana cerana*) larvae when exposed to mercury, implicating that encoded enzyme protects honeybees against heavy metals-mediated oxidative stress (Yu et al., 2012a). The transcript levels of Calcyclin binding protein (CacyBP) are expressed at the developmental stages in the honeybee brain, and are upregulated in response to oxidative stress induced by acetamiprid and $HgCl_2$, indicating that CacyBP protein is probably involved with the developmental regulation and the stress(Yu et al., 2012b). In addition, some members of the cytochrome P450 genes (CYP3 clade) involved with pesticide detoxification are downregulated in honeybees during pollination in greenhouses (Morimoto et al., 2011). Two CYP3 genes (6AQ1, 6BD1) expressed in the honeybee are orthologs of fruit fly CYP6G1 gene. CYP6G1 is associated with dichloro-diphenyl-trichloro-ethane and neonicotinoid resistance (Daborn et al. 2002). The downregulation of antiaoxidant genes in foragers may exacerbate the colony decline.

nAChRs in honeybee brain are a target of imidacloprid and imidacloprid-induced inhibition of the insect GABA receptor (Déglise et al., 2002). A slight activation of the cholinergic system with lower dose of imidacloprid applied on the thorax facilitates learning in honeybees (Lambin et al., 2001). Out of the six imidacloprid metabolites (5-hydroxyimidacloprid, 4,5-dihydroxyimidacloprid, desnitroimidacloprid, 6-chloronicotinic acid, olefin, and urea derivative) after 48 h treatment tested in *A. mellifera*, only two (5-hydroxyimidacloprid and olefin) exhibited chronic toxicity (hyperresponsiveness, hyperactivity, and trembling) close to that of imidacloprid, however, 72 h post- onset of intoxication all products resulted in bee mortality due to chronic activation (Suchail et al., 2001), implicating all metabolites differ in their efficacy and potency for nAChRs and when used for extended period of time show the chronic response. Consequently, another study reported the lowest effective concentrations (LOEC) for chronic feeding of pesticides (imidacloprid, 24 $\mu g\,kg^{-1}$; and 5-OH-imidacloprid, 120 $\mu g\,kg^{-1}$) to observe effect on the mortality of winter bees by monitoring their olfactory learning (Decourtye et al., 2003). The LOEC of imidacloprid was lower in summer bees (12 $\mu g\,kg^{-1}$) than in winter bees (48 $\mu g\,kg^{-1}$), indicating that the

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learning performances in *A. mellifera* are differentially affected by imidacloprid due to seasononal changes (Decourtye et al., 2003). The chronic activation of postsynaptic nAChRs by neonicotinoids causes persistent activation of receptors, which induces a rapid increase of Ca^{2+} influx into the neuron, resulting in impairment of cellular functions (Cassida and Quistad, 2004; Jeschke and Nauen, 2008).

Under physiological conditions, intracellular Ca^{2+} plays an important role in the formation of protein-dependent olfactory long-term memory in honeybees (Runckel et al., 2011). My hypothesis for CCD mechanism is that the chronic and synergistic interaction of pesticides (neonicotenoid/formamidine) with their receptors (in the hive or field) causes persistent activation of receptors, resulting in extensive influx of Ca^{2+} in the honeybee brain. Ca^{2+} influx produces accumulation of Ca^{2+} into the cytoplasm that is rapidly sequestrated by mitochondria. The mitochondrial Ca^{2+} overload consequently alters Ca^{2+} homeostasis that participates in mitochondrial dysfunction, resulting in increased mitochondrial permeability, increased mitochondrial ROS formation, uncoupling of mitochondrial electron transfer, decreased ATP production, and causing oxidative damage (Farooqui et al., 2008). Decrease in ATP/ADP ratio induces closure of cell-surface ATP-sensitive K^+ (K_{ATP}) channels, leading to cell membrane depolarization, followed by activation of many enzymes (such as phospholipase A_2, PLA_2; cyclooxygenase, COX; lipoxygenase, LOX; Ca^{2+}-calmodulin-dependent nitric oxide synthase, NOS; endonucleases and calpains) that damage cell macromolecules (Farooqui et al., 2008). Non-enzymic oxidation of ARA produces ROS, which activates NF-κB, a key regulator of neuronal death (Fig. 3). ROS interacts with NF-κB subunits in the cytoplasm and promotes its translocation from the cytoplasm to the nucleus (Farooqui, 2012b; Farooqui, 2008). NF-κB promotes the transcription of genes related to oxidative stress and inflammation, encoding number of proteins/enzymes related to oxidative stress and inflammation, damaging cholinergic/octopaminergic neurons (Farooqui, 2012b), resulting in olfactory dysfunction contributing to CCD.

8. Concluding remarks

The biogenic amines such as ACh and OCT are involved in the modulation of learning and memory in honeybees. However, increased concentration of biogenic amines in the honeybee brain can be highly toxic (acute intoxication) due to sudden and rapid influx of [Ca^{2+}], mitochondrial dysfunction, generation of excessive ROS, alteration in neural functions, impairment of various cognitive and non-cognitive behaviors as well as disturbance in other physiological actions, leading to death. Similarly, depending on the dose and duration (acute, subacute, subchronic and chronic), biogenic amines-based pesticides may elicit lethal, sublethal, subchronic and chronic effects of different natures on honeybees. Like ACh and OCT, biogenic amines-based pesticides such as neonicotinoids and formamidines exert agonistic action on their corresponding receptors. Chronic activation of biogenic amine receptors by biogenic amines-based pesticide (low dose, repeated exposures of pesticide to honeybees) will cause low but persistent activation of the receptor, resulting in increased influx of Ca^{2+}, an unbalanced overproduction of ROS and RNS may give rise to oxidative and nitrosative stress, inducing neuronal damage, resulting in impairement of cognitive functions such as decrease in olfactory learning and memory. Thus, acute activation of biogenic amine receptors by pesticides results in the rapid appearance of neurotoxicity symptoms, whereas chronic activation is a slow process, therefore honeybee mortality can be delayed for several days after the onset of intoxication. CCD is not caused by a single factor. It is now considered as a multifactorial phenomenon, reflecting the synergestic effect of multiple factors such as pesticides, infections, toxins, pathogens, parasites, nutritional stress and other other stresses. Thus, if honeybees are exposed to a mixture of pesticides and/or other environmental factors in the colony or in the field then synergistic interactions may aggravate ROS and RNS production, weaken immune system of honeybees, and disrupt olfactory learning and memory that may be a prominent reason of their failure in returning to their hives. Further investigations with biogenic amines-based pesticides are needed to prove this hypothesis.

In summary, biogenic amines-based pesticides, in particular neonicotenides, have been detected in pollen and nectar throughout the blooming period. With exception of few studies in which no effect of neonecotenoids was observed on bee mortality, many studies have reported that a synergistic interaction between neonicotinoid pesticides and other stressors likely contributes to CCD. Moreover, laboratory studies have successfully demonstrated that neonicotenides (alone or in combination with other factors) cause disorientation, reduced communication, impaired learning and memory, reduced longevity, and reduced feeding, which strongly support that neonicotenides may be one of the major factor involved with the onset of CCD phenomenon. It is timely that U.S. EPA's Office of Pesticide Programs and other countries which are still using systemic neonicotinoids should take an urgent action about regulatory policies and promptly suspend their use because synergistic and chronic effects can be highly toxic to honeybees as well as to other pollinating insects.

Conflict of interest statement

The author does not have any conflict of interest to declare.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neuint.2012.09.020.

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