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Non-neuronal acetylcholine involved in reproduction in mammals and honeybees

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Abbreviations:

ACh acetylcholine; CaMKII Ca^{2+} calmodulin-dependent protein kinase II; ChAT choline acetyltransferase; iPS induced pluripotent stem cells; mAChRs muscarinic receptors; nAChRs nicotine receptors; VAChT vesicular acetylcholine transporter

ABSTRACT:

Bacteria and archaea synthesize acetylcholine (ACh). Thus it can be postulated that ACh was created by nature roughly 3 billion years ago. Therefore, the wide expression of ACh in

nature (i.e. in bacteria, archaea, unicellular organisms, plants, fungi, non-vertebrates and vertebrates and the abundance of non-neuronal cells of mammals) is not surprising. The term non-neuronal ACh and non-neuronal cholinergic system have been introduced to describe the auto- and paracrine, i.e. local regulatory actions of cells not innervated by neuronal cholinergic fibers to communicate among themselves. In this way non-neuronal ACh binds to the nicotinic or muscarinic receptors expressed on these local and migrating cells and modulates basic cells functions such as proliferation, differentiation, migration and the transport of ions and water. The present article is focused to the effects of non-neuronal ACh linked to reproduction; data on the expression and function of the non-neuronal cholinergic system in the following topics are summarized: 1 Sperm, granulosa cells, oocytes; 2 Auxiliary systems (ovary, oviduct, placenta); 3 Embryonic stem cells as first step for reproduction of a new individual after fertilization; 4 Larval food as an example of reproduction in insects (honeybees) and adverse effects of the neonicotinoids, a class of world-wide applied insecticides. The review article will show that non-neuronal ACh is substantially involved in the regulation of reproduction in mammals and also non-mammals like insects (honeybees). There is a need to learn more about this biological role of ACh. In particular, we have to consider that insecticides like the neonicotinoids, but also carbamates and organophosphorus pesticides, interfere with the non-neuronal cholinergic system thus compromising for example the breeding of honeybees. But it is possible that other species may also be adversely affected as well, a mechanism which may contribute to the observed decline in biodiversity.

INTRODUCTION

In the last century basic research has demonstrated the ubiquitous expression of acetylcholine (ACh) in nature, i.e. in bacteria, archaea, unicellular organisms, plants, fungi, non-vertebrates and vertebrates and mammals (Sastry and Sadavongvivad, 1979; Grando, 1997; Wessler *et al.*, 1998, 1999, 2001b; Wessler and Kirkpatrick, 2008). Thus, ACh was created by nature roughly 3 billion years ago, when bacteria and archaea populated our probiotic earth (Fig. 1). Therewith, it becomes evident that on the evolutionary time scale, ACh represents one of the oldest signaling molecules, i.e. established at a time when life was evolving in our world (Sastry and Sadavongvivad, 1979; Wessler *et al.*, 1999; Horiuchi *et al*., 2003; Yamada *et al*., 2005). Both bacteria and archaea are regarded as the starting points of the universal

phylogenetic tree to establish the higher developed eukaryotes. Thus, the wide expression of ACh and the cholinergic system in nature is not a surprising finding. In addition, one can argue that ACh represents an ester of a very common and abundant base and acid, a property which facilitates its wide expression in nature. Today it is not known whether ACh mediates regulatory processes in bacteria and other non-metazoans. Nevertheless, some bacteria also express cholinesterase allowing at least different levels of ACh (Domenech *et al.*, 1981). In plants clear regulatory roles of ACh have been described. For example, in urtica dioica ACh is involved in the regulation of water homeostasis and photosynthesis, effects which are blocked by classical receptor antagonists like atropine and tubocurarine (Wessler *et al.*, 2001b).

When neuronal cells were at first established in marine organisms nearly 2.5 billon years later, these cells took advantage of the already existing cholinergic system including ACh but have created the vesicles, the vesicular ACh transporter VAChT, synapses or junctional specialization with hot spots for the receptors and esterase to mediate rapid communication on the ms time scale (Fig. 1). Thus, non-neuronal ACh has been used as the model for neuronal ACh transmitting signals generated by neurons. To discriminate between both systems the terms non-neuronal ACh and non-neuronal cholinergic system have been introduced to describe the expression and biological role of ACh released from non-neuronal cells to communicate with neighboring non-neuronal cells and also to trigger intracellular signaling independent of neuronal input (Wessler *et al*., 1998, 1999). The roles of the colinesterases differ between both systems. Within the neuronal cholinergic system, the enzymatic action of cholinesterases to terminate the signaling of the neurotransmitter ACh is dominant, whereas in the non-neuronal system multiple, also non-enzymatic effects of cholinesterases have been discussed. For example, embryogenesis, ossification, proliferation, apoptosis, angiogenesis and the function of the cardiovascular and immune system can be modified by the activity or the expression levels of cholinesterases (Layer and Willbold, 1995; Bicker *et al*., 2004; Thullbery *et al.,* 2005;Silman and Sussmann, 2005; Santos *et al*., 2007; Kakinuma *et al.,* 2012; Tsim and Soreq, 2013; Roy *et al.*, 2015). Based on these findings the biological role of ACh and the cholinergic system has been revisited during the last 20 years. ACh and the pivotal components of the cholinergic system (high affinity choline uptake, choline acetyltransferase and its end product ACh, muscarinic and nicotinic receptors, esterase) are expressed by more or less all mammalian cells, i.e. cells not innervated by neurons at all. Via auto- and paracrine modes of action non-neuronal ACh

promotes cell proliferation and differentiation as well as regulation of cell-cell contact, locomotion, and transport of ions and water. The present article will focus on the contribution of the non-neuronal cholinergic system to the reproductive system, i.e. to the generation of individual life. Thus, the expression and function of the non-neuronal system is described with respect to the following topics: 1. Sperm, granulosa cells, oocytes. 2. Auxiliary systems (ovary, oviduct, placenta). 3. Embryonic stem cells as first step for reproduction of a new individual after fertilization. 4. Larval food as an example of reproduction in insects (honeybees) and adverse effects of neonicotinoids.

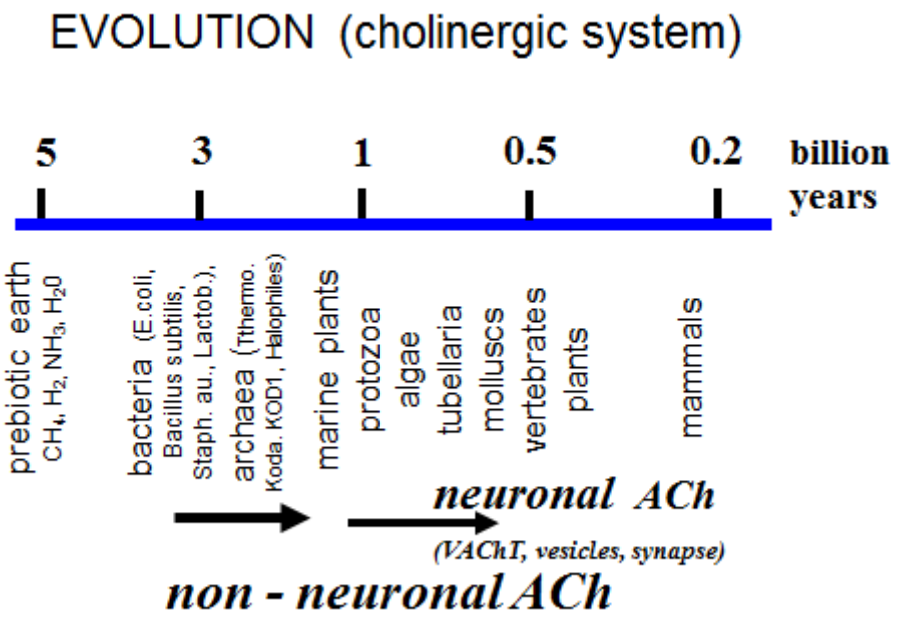

Fig. 1: Evolutionary time scale for ACh

ACh is synthesized by bacteriae and archaea (Sastry and Sadavongvivad, 1979; Wessler *et al*., 1999; Horiuchi *et al*., 2003; Yamada *et al.,* 2005). Both micro-organisms are regarded as the starting point of the common phylogentic tree, approximately 3 billion years ago. Two and a half billion years later the first neuronal cell appeared in marine organisms. These neuronal cells took advantage of the already established non-neuronal cholinergic system and further optimized the system to mediate rapid communication on the ms time scale by the generation of vesicles, the vesicular transporter VAChT and the formation of synapses or junctional endings with hot spots of esterase and the respective nicotinic and muscarinic

receptors. In this context it should be considered that the role of cholinesterases differs between metazoan and non-metazoan systems like bacteria, plants and fungi. Abbr.: E. coli. Escherichia coli; Staph. au. Staphylococcus aureus; Lactob. Lactobacillus; Thermo. Koda KOD1 Thermococcus kodakaraensis KOD1; Halophiles Halophiles Halobacterium sp. NRC-1.

1. Sperm, granulosa cells, oocytes

ACh has been detected in the sperm of rabbit, bull, and man (Saiko, 1969; Bishop *et al*., 1977). In chemotaxis assays it was found that low doses of ACh increased migration of mouse spermatozoa and that α-bungarotoxin (nAChR antagonist) blocked human sperm motility more or less completely (Jaffe, 1990; Sliwa, 1995). Likewise, inhibition of the synthesizing enzyme, the choline acetyltransferase (ChAT), reduced human sperm motility substantially (Sastry *et al*., 1981). Using immunofluorescent or immunogold staining muscarinic receptor binding was identified mainly in the head region, whereas nicotinic receptor binding was visualized in the tail and post-acrosomal area (Baccetti *et al*., 1995). Later on, in human sperm the expression of the α3, α5, α7, α9 and ß4 nicotine receptor subunits was demonstrated (Kumar and Meizel, 2005).

Oocytes of some species have been found to synthesize ACh and express muscarinic receptors; moreover, in monkey and human oocytes the expression of M3 has been described (Eusebi *et al*., 1984; Fritz *et al*., 2001; Angellini *et al*., 2004). The fertilized oocytes of the honeybees also contain ACh (Wessler *et al*., 2016). Human granulosa cells have been shown to express the mRNA for M1 and M5 (Fritz *et al*., 2001).

The acrosome of the spermatozoa is located in the anterior region of the sperm head and represents a secretory vesicle. Upon fertilization this area of the sperm head must perform the acrosome reaction, i.e. a so-called exocytotic event between the plasma membrane of the sperm head and the underlying membrane which allows binding to the zona pellucida of the oocyte. The acrosome reaction is triggered by a glycoprotein of the zona pellucida which induces the opening of a low voltage-sensitive Ca^{2+} channel and a transient receptor potential protein cation channel (Meizel and Son, 2005). Both channels mediate the Ca^{2+} influx required for the acrosome reaction and the fusion between sperm and egg. It has been demonstrated that in mouse and human sperm the α7 subunit is involved in triggering the

cation influx required for the acrosome reaction of the sperm (Bray *et al*., 2002; Son and Meizel, 2003; Meizel and Son, 2005). Thus, the non-neuronal cholinergic system regulating multiple steps like sperm motility and acrosome reaction is directly involved in the process of fertilization and reproduction. In line with this, it has been shown that ACh applied to human sperm caused an increase of intercellular calcium (Bray *et al*., 2005a). When ACh (10-250 µM) is exposed to capacitated human sperm in combination with soluble zona pellucida protein, the acrosome reaction is induced, and this reaction is reduced by α-bungarotoxin (Bray *et al*., 2002, 2005a). Finally, it has been reported that mice deficient in CHRNA7 showed reduced receptor binding at the sperm midpiece and reduced sperm motility (Bray *et al*., 2005b)

2. Auxillary systems (testes, ovary, oviduct, placenta)

The non-neuronal cholinergic system is widely expressed within the male rat reproductive tract. For example, mAChRs (M1, M2, M3) are expressed within the efferent ductules and the epididymidis. Expression of various subunits of nAChRs, ChAT, choline-transporters and the vesicular ACh transporter have been demonstrated in the parenchyma of rat testes within an area not innervated by the autonomic nervous system (Avellar *et al*., 2010; Schimer *et al*., 2011). Stimulation by ACh causes increased vasoactivity, muscle contraction, sperm transport and cell proliferation (Hodson, 1965; Kumazawa *et al*., 1987; Luthin *et al*., 1997; Lau *et al*., 2000; Avellar *et al*., 2010; Schirmer *et al*., 2011). In animal studies antagonists of mAChRs impaired fertility, probably because of a compromised transport of sperm and semen from the vas deferens and seminal gland to the urethra during emission (Ban *et al*., 2002; Sato *et al*., 2005). Expression of the nAChRs subunits differed between the Sertoli cells, spermatogonia and spermatocytes, thus indicating a potential role of non-neuronal ACh in germ cell differentiation (Schirmer *et al*., 2011).

In addition, the female reproductive system expresses the non-neuronal cholinergic system. The ovarian endocrine cells of rodents and primates, i.e. the granulosa cells, express the synthesizing enzyme ChAT, contain ACh detected by HPLC combined with bioreactors and also express mAChRs (M1, M5; Mayerhofer *et al*., 2003; Mayerhofer and Kunz, 2005). Human cultured granulosa cells synthesize ACh (4-11 pmol/10^6 cells; Fritz *et al*. 2001). Interestingly, the synthesis of ACh occurs in the growing follicle which was stimulated by FSH (Fritz *et al*. 2001). Later on ACh-mediated stimulation of the muscarinic receptors

causes activation of the transcription factor erg-1, intracellular calcium increase and activation of voltage-dependent ion channels, altered steroidogenesis and disruption of intercellular cell communication. This facilitates the separation of granulosa cells from the functional syncytium, proliferation and locomotion (Mayerhofer and Kunz, 2005). Therewith, a highly specialized regulatory pathway to attain a distinct cell function is mediated by the local cell transmitter ACh. Moreover, the porcine oviductal epithelium expresses ChAT and synthesizes ACh (Steffl *et al*., 2006). Interestingly, the degree of ChAT-expression measured by immunohistochemistry markedly decreased during dioestrus and prooestrus stages but increased during early pregnancy, showing a hormone-sensitive regulation (Steffl *et al*., 2006). Recently, a new non-enzymatic effect of cholinesterase has been detected in ovarian cells. Via RIPK1/MLKL-dependent pathway (serine/threonine kinase 1 pseudokinase substrate mixed lineage kinase-like) this protein regulates cell necrosis (necroptosis) and therewith the life and death of ovarian cells (Blohberger *et al*., 2015). Thus, the non-neuronal cholinergic system appears to be closely involved in the regulation of the proliferation, differentiation and death of ovarian cells.

The placenta, an organ not innervated by extrinsic or intrinsic cholinergic neurons, has been intensively characterized with respect to its expression of the non-neuronal cholinergic system. Nearly 100 years before the occurrence of ACh in the human placenta was demonstrated in the late 1920s (Loewi and Navratil 1926) and later on, Bischoff and Haupstein confirmed this finding (Bischoff *et al*., 1932; Haupstein, 1932). Meanwhile it has been shown that the human placenta (chorionic plate, villus region, basal plate) expresses the complete cholinergic system, i.e. the synthesizing enzyme choline acetyltransferase, m- and nAChRs and the degrading enzyme cholinesterase (Sastry and Olubadewa, 1976; Sastry and Sadavongvivad 1979; Sastry 1997). Release of ACh has been measured from isolated villus pieces or in vitro perfused placental cotyleda (Olubadewo and Sastry 1978; Boura *et al*. 1986; Sastry 1997). In addition, the release mechanism for non-neuronal ACh has partly been clarified with ACh being transported via organic cation transporters (Wessler *et al*., 2001a). The amniotic epithelial membrane cells and the amniotic fluid also contain ACh (Horikoshi *et al*., 2003). MAChR subtypes were found almost exclusively in syncytiotrophoblast, but nAChRs are also expressed in the placenta (Sastry, 1997).

In experimentally induced intrauterine growth retardation some increase of ACh in the amniotic fluid was observed (Horikoshi *et al.*, 2003). However, our knowledge of the physiological role of the non-neuronal cholinergic system within the placenta is very limited.

Probably ACh may be required for regulation of villus movements. The following regulatory functions have been attributed to ACh released from the mammalian placenta (Sastry, 1997): (a) regulation of blood flow and fluid volume in placental vessels; (b) regulation of the activity of trophoblastic channels; (c) activation of myofibroblasts; (d) modulation of amino acid transport; (e) release of placental hormones and mediators. For example, it has been demonstrated that via mAChRs on the trophoblast cell membrane ACh modulates the NO- generation in an estrogen-dependent manner (Bhuiyan *et al.*, 2006). In conclusion it is obvious that the non-neuronal cholinergic system within the placenta of mammals is closely involved in the regulation of embryogenesis.

3. Embryonic stem cells (murine CGR8 cells) and iPS cells

Embryonic stem cells or induced pluripotent stem cells (iPS cells) are capable of differentiating into cells of all three germ layers of the embryo including neuronal cells and are regarded as a possible substitute for degenerative diseases. Therefore, these cells attain increasing scientific interest and therapeutic significance. ACh has been demonstrated in murine embryonic stem cells (Paraoanu *et al*., 2007). Release of ACh from these stem cells is partly mediated by organic cation transporters (Wessler *et al*., 2012). Moreover, it has been shown that the murine CGR8 stem cell line expressed all subtypes of mAChRs and more or less all nAChRs subunits (α3, α4, α7, α9, α10, β1, β2, β3, β4, γ, δ, ε; Kaltwasser *et al*., 2015). In addition, mouse iPS cells express nAChRs (α4, α7) and stimulation of these nAChRs caused a significant increase of mouse iPS cell proliferation through a CaMKΠ signaling pathway (Ishizuka *et al*., 2012). The human iPS cell line ATCC-DYP0250 also expressed mAChRs (M2, M3, M4) and nAChRs subunits (α3, α4, α9, α10, β1, β2, β4, γ; Wessler, 2016).

Meanwhile, there exists clear evidence that non-neuronal ACh mediates a functional role within murine embryonic stem cells. Thus, for example, nicotine stimulates the expression of the transcription factors Oct-4 and Rex-1 (Zhang et al., 2005). Experimental hypoxia limits DNA synthesis and increases apoptosis but the application of exogenous ACh prevented these cell-damaging effects in an atropine-sensitive manner (Kim *et al*., 2008). The cholinergic system is also involved in the regulation of the complex events occurring, when

pluripotent cells undergo first differentiation and become totipotent. At this stage the ChAT enzyme activity and ACh cell content increases and the expression of α4 and β4 is markedly down regulated in CGR8 cells (Wessler *et al*., 2013; Kaltwasser *et al*., 2015). All these findings represent thus far only fragmented pieces of knowledge about the regulatory role of non-neuronal ACh in stem cells. Therefore, the complete picture regarding the significance of the non-neuronal cholinergic system in controlling – together with other mediators - the phenotypic functions of embryonic stems cells has to be illuminated in the future.

4. Larval food and breeding of honeybees and adverse effects of neonicotinoids

Already in 1960 it was reported that the royal jelly, the secretion product of nursing bees to feed their queen and the food for queen larvae of honeybees, contains considerable amounts of ACh (Colhoun and Smith, 1960). However, the authors questioned the existence of ACh, because they did not detect any corresponding ChAT activity in the glandular tissue of the honeybees. Very recently, the existence of ACh in royal jelly and the larval food obtained from bees nursing working bee larvae has been confirmed by HPLC measurement combined with bioreactors and electrochemical detection (Wessler *et al*., 2016). High concentrations between 5 and 8 mM ACh were detected in freshly isolated royal jelly, but also in the larval food applied to working bee larvae and in commercially available royal jelly (Wessler *et al*., 2016). The royal jelly/larval food is produced in the hypopharyngeal gland of nursing bees, which is localized below the brain. This gland consists of the secretory buds containing the secretory acini, the main collecting duct and smaller ducts and microtubes connecting the secretory buds with the main collecting duct.

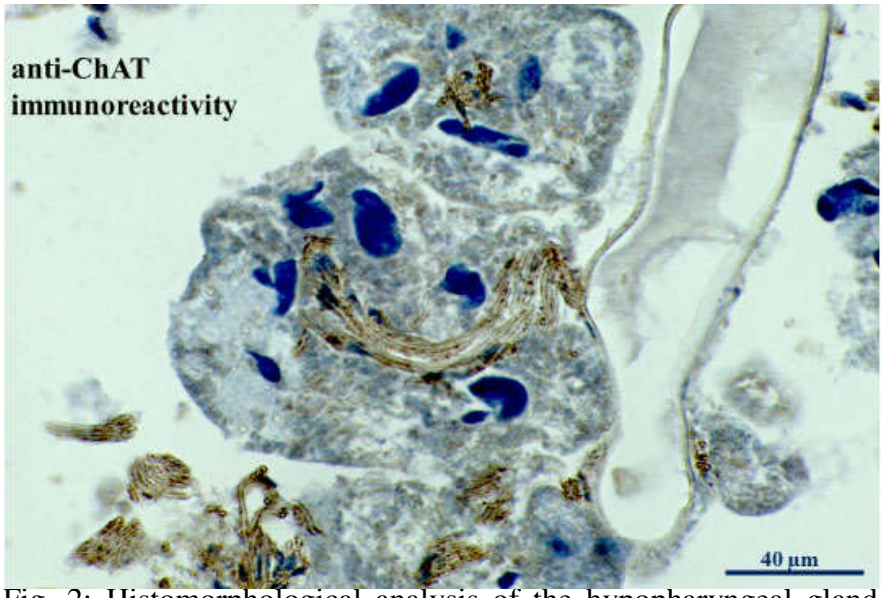

Fig. 2: Histomorphological analysis of the hypopharyngeal gland by anti-ChAT immuno-histochemistry. A standard protocol was used and the following antibodies were applied: rabbit anti-ChAT antibody (Abcam ab 68779, UK; 1:400 dilution; Envision Flex+rabbit, Dako K8019, Germany, used as second antibody). Histology was evaluated and documented using a Keyence BZ-9000E Hs all-in-one microscope (Keyence, Germany). Secretory acini with cytosol and nuclei (blue staining) and a tangentially cut duct are shown. The fine long distance microtubes of the so-called canal cells (nuclei not visible), which open into the collecting duct, express prominent anti-ChAT immunoreactivity (brown staining in tangentially and horizontally cut microtubes).

When ChAT enzyme activity of the hypopharyngeal gland was measured, a high ACh synthesis rate (2.2±0.80 µmol/mg/h; Wessler *et al*., 2016) was obtained only in the absence of the detergent Tween 100, thus indicating the existence of a membrane-bound protein. ChAT lossed its enzymatic activity after detaching the enzyme from its embedding membrane. This finding correlates excellently with the morphological images localizing the enzyme within the wall of the microtubes and the wall of the collecting duct (Wessler *et al*., 2016).

Nursing honeybees have developed a highly effective method to deliver 100 % of the synthesized ACh to the larvae. The pH of royal jelly/larval food is 4 and therewith ACh is

protected from any spontaneous hydrolysis and esterase activity. Both enzymes, acetylcholinesterase and butyrylcholinesterase, operate optimally at a pH of 7.4 but are acid-labile. Thus, at a pH below 5 enzyme activity is lost completely. Therewith, ACh synthesized by the wall-cells of the microtubes and added as a final step to the royal jelly/larval food will be completely delivered to the larvae placed within the brood combs. Royal jelly can be treated by adding both alkali to increase the pH to 5.5 and butyrylcholinesterase to remove ACh. Using this severely manipulated food in artificial larvae breeding experiments it was found that the survival rate of larvae was significantly lower compared to the same manipulated food which was supplemented with 6 mM ACh just prior to the application (Wessler *et al*., 2016). This finding indicates the significance of ACh for larval breeding, i.e. for the reproduction of honeybees, a non-mammalian organism. This is consistent with the proliferative and so-called trophic effects of ACh mediated via n- and mAChRs as has been repeatedly demonstrated elsewhere (Sastry and Sadavongvivad, 1998; Schuller *et al*., 1990 Catone and Ternaux, 2003; Wessler and Kirkpatrick, 2008; Grando, 2014).

In this context it is important to consider that our knowledge about a possible role of ACh and the cholinergic system in reproduction processes of non-mammalian animals is very scanty. It is likely that multiple species have made use of the signaling and trophic properties of ACh and the cholinergic system to regulate their own reproduction. Even plants show such comparable mechanisms. For example, ACh or nicotine induces rooting and promotes secondary root formation in leaf explants of tomato (*Lycopersicon esculentum Miller var. Pusa Ruby*; Bamel *et al*., 2015). Therewith, the generation of fruit and seed will become facilitated. It is important to clarify the role of ACh in all these systems for the following reason. Most of the world-wide used pesticides interfere with the cholinergic system, for example the carbamates, the esterase inhibitors (organophosphorus pesticides) and the neonicotinoids.

The neuroactive neonicotinoids interfere with the cholinergic system by binding to the insect nicotinic receptors (Matsuda *et al*., 2001). Although a causal relationship between bee losses and the widespread use of neonicotinoids in agriculture is still under debate, several studies discuss them as possible contributing factors for colony losses without a causative mechanistic explanation (Vidau *et al.*, 2001; Henry *et al*., 2012; Gill *et al.,* 2012; Whitehorn *et al*., 2012; Goulson *et al.*, 2015; Rundlof *et al.*, 2015). The increasing concern about their risks on bee health has led to an EU-wide ban by the European Commission on three commonly used neonicotinoids (clothianidin, imidacloprid, thiamethoxam). Recently,

adverse effects of neonicotinoids on the synthesis of ACh in the hypopharyngeal gland of the nursing bees have been described (Wessler *et al.*, 2016). Within this gland the α3-and α4-subunit of nAChRs are expressed. Chronic exposure of honeybee colonies to low field-relevant concentrations of 200 ppb thiacloprid caused a significant reduction of the ACh-concentration of the larval food (Wessler *et al*., 2016). Likewise, when honeybees were fed with low concentrations of chlothianidin (1 and 10 ppb) the amount of ACh in the larval food was reduced, a condition which compromised larval breeding (Wessler *et al*., 2016). At higher concentrations (100 ppb chlothianidin or 8,800 ppb thiacloprid) the neonicotinoids caused significant damage to the hypopharyngeal gland with vacuolization of the secretory cells and even loss of complete secretory buds (Wessler *et al*., 2016). These experiments provide evidence for a direct negative input of the world-wide applied insecticides on the reproduction of honeybees by interacting with the non-neuronal cholinergic system. At this time, the regulatory authorities have the responsibility of taking the new findings into account when reassessing the benefit-risk ratio of the neonicotinoids. In particular, the permitted residual concentrations have to be reduced worldwide to minimize environmentally adverse effects.

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The authors declare no conflicts of interest.

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