Nicotinic Receptors of Parasitic Insects: Biochemical and Pharmacological Studies

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Für meinen Mann
w wiecznej wdzięczności i miłości

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ABBREVIATIONS

µA	microampere
µM	micromolar
^{1}H-NMR	proton nuclear magnetic resonance
5-HT3	5-hydroxytryptamine receptor 3 family
Å	Ångström
ACh	acetylcholine
AChBP	acetylcholine-binding protein
AChR	acetylcholine receptor
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
cRNA	complementary ribonucleic acid
Cys	cysteine
EC_{50}	effective concentration 50 = agonist concentration achieving 50% maximum response
e.g.	exempli gratia = for example
Fig.	figure
GABA	γ-aminobutyric acid
I_{max}	maximum current response measured at its peak in the experiment
kDa	kilodalton
LGIC	ligand gated ion channel
mM	millimolar

nA	nanoampere
nAChR	nicotinic acetylcholine receptor
nM	nanomolar
PCR	polymerase chain reaction
pH	decimal logarithm of the reciprocal of the hydrogen ion activity
rac-	racemic mixture
RACE-PCR	rapid amplification of cDNA ends PCR
RNA	ribonucleic acid
RT-PCR	reverse transcription PCR
S	serine
TM2	the second of the transmembrane regions
Y	tyrosine

SUMMARY

Nicotinic acetylcholine receptors (nAChRs) are ligand-controlled cation channels, which act in fast neurotransmission at cholinergic synapses in vertebrates and invertebrates. They are the binding sites for nicotinoid drugs, such as nicotine and epibatidine. Insect nicotinic acetylcholine receptors are targets of several insecticide classes, such as the neonicotinoids, spinosyns and nereis toxins. This study is the first report about the gene identification of the α1 and α2 subunits (Lcα1 and Lcα2) from the sheep blowfly *Lucilia cuprina* as well as the full length cDNA cloning of these two subunits, and of the three *Ctenocephalides felis* (cat flea) nAChR α subunit genes Cfα1, Cfα2, and Cfα3 previously not available as full length versions. Expression of these subunits in *Xenopus laevis* oocytes as hybrid receptors with the *Gallus gallus* (chicken) β2 nAChR (Ggβ2) subunit resulted in functional acetylcholine-responsive ion channels, as judged from our voltage clamp experiments. Cfα2/Ggβ2 and Lcα2/Ggβ2 proved to be insensitive to α-bungarotoxin, while acetylcholine-induced currents of the Cfα1/Ggβ2 and Lcα1/Ggβ2 combinations were completely blocked by this snake toxin. These characteristics of α-bungarotoxin sensitivity have been considered hallmarks of the α1 and α2 gene families and are confirmed here for two additional examples. The pharmacological profiles of Cfα1/Ggβ2, Cfα2/Ggβ2 and the chicken neuronal receptor Ggα4/Ggβ2 for acetylcholine, two nicotinoids and 6 insecticidal neonicotinoids were determined and compared on the basis of EC_{50}, Hill coefficient and maximal current (relative to acetylcholine, I_{max}). Particularly remarkable was the finding that Cfα1/Ggβ2 was far more sensitive to acetylcholine, nicotine and neonicotinoid agonists than either Cfα2/Ggβ2 or Ggα4/Ggβ2: for the anti-flea neonicotinoid market compound imidacloprid the respective EC_{50} values were 0.02 μM, 1.31 μM and 13.8 μM. These results were also confirmed for two other insect species, *Drosophila melanogaster* and *Lucilia cuprina*, where the pharmacological profile of the Dmα1, Dmα2, Lcα1 and Lcα2 subunits as hybrid receptors with Ggβ2 in *Xenopus* oocyte expressions resulted in similar sensitivity patterns as those identified for the *Ctenocephalides felis* orthologs. For Cfα3/Ggβ2, functional expression could be achieved, but detailed analysis of acetylcholine and other agonists used in this study could not be performed in electrophysiological

experiments, due to the low signals. Collectively, the results of this study show that at least in a Ggβ2 hybrid receptor setting, with respect to EC_{50}, insect α1 subunits confer a 9 to 65 fold higher sensitivity to neonicotinoids than seen with α2 subunits, which may contribute *in vivo* to the insect-selective action of this pesticide class. In an attempt to elucidate ligand structure-activity relationships, eight close derivatives of acetylcholine were chemically synthesized and, together with five purchased compounds, analysed in voltage clamp experiments for Lcα1/Ggβ2, Lcα2/Ggβ2 and Ggα4/Ggβ2. Comparison of the data for insect *versus* chicken nAChR α subunits allowed the definition of novel structure-activity and structure-selectivity relationships. In the case of *N*-ethyl-acetylcholine, the EC_{50} value of the chicken Ggα4/Ggβ2 was increased almost by a factor of 1000, while for both Lcα1/Ggβ2 and Lcα2/Ggβ2, potency remained similar to that of acetylcholine. Further derivatives with strong insect nAChR selectivity potential were acetyl-α-methylcholine and trimethyl-(3-methoxy-3-oxopropyl) ammonium, followed by acetylhomocholine and trimethyl-(4-oxopentyl) ammonium. Identification of these insect-specific structure-activity relationship features may provide guidance for identification or design of insect nAChR agonists by structure-based or *in silico* methods.

ZUSAMMENFASSUNG

Nikotinische Acetylcholinrezeptoren (nAChRs) gehören zu den ligandengesteuerten Kationenkanälen, welche in der schnellen neuronalen Übertragung an cholinergen Synapsen der Vertebraten und Invertebraten mitwirken. Sie binden nikotinoide Wirkstoffe wie Nikotin und Epibatidin. Nikotinische Acetylcholinrezeptoren von Insekten sind Angriffspunkte von einigen Insektizidklassen, wie Neonikotinoide, Spinosyne und Nereistoxine. Diese Arbeit beschreibt zum ersten Mal die Genidentifizierung der α1 und α2 Untereinheiten (Lcα1 und Lcα2) der Schaf-Schmeissfliege *Lucilia cuprina* und die cDNA-Klonierung dieser zwei Untereinheiten, wie auch der drei *Ctenocephalides felis* (Katzenfloh) nAChR α-Untereinheiten Cfα1, Cfα2 und Cfα3, deren vollständige Gensequenz bis dahin noch nicht bekannt war. Die Expression der Untereinheiten in *Xenopus laevis* Oozyten als hybride Rezeptoren zusammen mit der *Gallus gallus* (Huhn) β2 nAChR (Ggβ2) Untereinheit führten zur Ausbildung von Acetylcholin-gesteuerten Ionenkanälen, was durch Voltage-Clamp Experimente bestätigt wurde. Cfα2/Ggβ2 und Lcα2/Ggβ2 waren insensitiv gegenüber α-Bungarotoxin, während Acetylcholin-induzierte Ströme von Cfα1/Ggβ2 und Lcα1/Ggβ2 durch das Schlangengift komplett blockiert werden konnten. Diese Merkmale der α-Bungarotoxin-Antwort wurden als kennzeichnend für die α1 und α2 nAChR-Genfamilien betrachtet und in dieser Arbeit für zwei weitere Beispiele bestätigt. Die pharmakologischen Profile von Cfα1/Ggβ2, Cfα2/Ggβ2 und vom neuronalen Rezeptor des Huhns Ggα4/Ggβ2 für Acetylcholin, zwei Nikotinoide und 6 insektizide Neonikotinoide wurden auf der Basis vom EC_{50}, Hill-Koeffizienten und maximalen Strom (relativ zu Acetylcholin, I_{max}) bestimmt und miteinander verglichen. Besonders bemerkenswert war die Erkenntnis, dass Cfα1/Ggβ2 wesentlich sensitiver gegenüber Acetylcholin, Nikotin und Neonikotinoide war als Cfα2/Ggβ2 oder Ggα4/Ggβ2: für das Anti-Floh-Neonikotinoid Imidacloprid waren die jeweiligen EC_{50}-Werte 0,02 μM, 1,31 μM und 13,8 μM. Diese Ergebnisse konnten ebenfalls für zwei weitere Insekten-Species, *Drosophila melanogaster* und *Lucilia cuprina*, bestätigt werden: die pharmakologischen Profile von Dmα1, Dmα2, Lcα1 und Lcα2 Untereinheiten exprimiert als Hybridrezeptoren zusammen mit Ggβ2 in *Xenopus*-Oozyten ergaben ähnliche Sensitivitätmuster, wie die für die *C. felis*-

Orthologen identifizierten. Cfα3/Ggβ2 konnte zwar funktionell exprimiert werden, aufgrund nur kleiner Signale war allerdings eine detaillierte elektrophysiologische Analyse von Acetylcholin und anderer Agonisten, die hier untersucht wurden, nicht möglich. Insgesamt zeigen die Ergebnisse dieser Arbeit, dass, zumindest im Rahmen eines Hybridrezeptors zusammen mit Ggβ2, Insekten α1 Untereinheiten eine 9 bis 65 fache höhere Sensitivität in Bezug auf EC_{50}-Werte gegenüber Neonikotinoiden aufweisen als α2 Untereinheiten. Dies könnte die insektenselektive Wirkung, die bei dieser Pestizidklasse *in vivo* beobachtet wird, erklären. In einem Versuch die Liganden-Struktur-Aktivitätsbeziehungen aufzuklären, wurden 8 Strukturanaloga von Acetylcholin chemisch synthetisiert und zusammen mit 5 kommerziell erworbenen Verbindungen in Voltage-Clamp Experimenten mit Lcα1/Ggβ2, Lcα2/Ggβ2 und Ggα4/Ggβ2 analysiert. Ein Vergleich der Daten für Insekten *versus* Huhn nAChR α Untereinheiten ermöglichte es neue Struktur-Wirkungs- und Struktur-Selektivitätsbeziehungen zu erkennen. Im Fall von *N*-Ethyl-Acetylcholin erhöhte sich der EC_{50}-Wert für Huhn Ggα4/Ggβ2 nahezu um den Faktor 1000 relativ zu ACh, während sowohl für Lcα1/Ggβ2 als auch für Lcα2/Ggβ2 die Wirksamkeit bei einem ähnlichen Wert blieb wie der von Acetylcholin. Weitere Derivate mit deutlichem Selektivitätspotential gegenüber Insekten-nAChR waren Acetyl-α-Methylcholin und Trimethyl-(3-Methoxy-3-Oxopropyl) Ammonium, gefolgt von Acetylhomocholin und Trimethyl-(4-Oxopentyl) Ammonium. Die Identifizierung dieser insektenspezifischen Eigenschaften der Struktur-Aktivitätsbeziehungen könnte eine Orientierung zur Identifizierung oder zum Design von Agonisten der Insekten-nAChR mithilfe strukturbasierter oder *in silico* Methoden liefern.

LIST OF PUBLICATIONS

1. Dederer H, Werr M, Ilg T. 2011. Differential sensitivity of *Ctenocephalides felis* and *Drosophila melanogaster* nicotinic acetylcholine receptor α1 and α2 subunits in recombinant hybrid receptors to nicotinoids and neonicotinoid insecticides. *Insect Biochem Mol Biol* 41(1):51-61.

2. Dederer H, Berger M, Meyer T, Werr M, Ilg T. 2013. Structure activity relationships of acetylcholine derivatives with *Lucilia cuprina* nicotinic acetylcholine receptor α1 and α2 subunits in chicken β2 subunit hybrid receptors in comparison with chicken nicotinic acetylcholine receptor α4/β2. *Insect Mol Biol* 22(2), 183-198.

CONTRIBUTIONS TO PUBLICATIONS

The scientific research and manuscript drawing were carried out under the supervision of Prof. Dr. Thomas Ilg at MSD Animal Health Innovation GmbH in Schwabenheim an der Selz, Germany. In the list below, my independent experimental work that led to the two publications is specified.

- Extraction of total RNA from *Lucilia cuprina* heads
- Identification and isolation of nAChR subunit genes α1 and α2 from *Lucilia cuprina* head total RNA: RT-PCR with degenerate primer pairs, RACE- and nested RACE-PCR, primer generation for RACE- and full length PCR, cloning and sequence analysis of PCR products, full length gene cloning
- Chemical synthesis of following acetylcholine derivatives: acetyl-*N*-ethylcholine, acetyl-*N*-propylcholine, acetyl-α-methylcholine, trimethyl-(4-oxopentyl) ammonium, trimethyl-(pentyl) ammonium, acetylhomocholine, trimethyl-(3-methoxy-3-oxopropyl) ammonium
- 5´-capped polyadenylated copy RNA synthesis of all nAChR subunit genes, injection of these cRNAs in *Xenopus laevis* oocytes for voltage clamp experiments
- Voltage clamp experiments and data analysis for the following hybrid nAChRs and compounds:
    - Cfα1/Ggβ2: EC_{50} values, Hill coefficients and I_{max} values for acetylcholine, nicotine, imidacloprid, nitenpyram, acetamiprid, dinotefuran and clothianidin; current response tests with α-bungarotoxin and thiacloprid
    - Cfα2/Ggβ2: EC_{50} values, Hill coefficients and I_{max} values for nicotine and epibatidine, I_{max} values for imidacloprid, nitenpyram, acetamiprid, dinotefuran and clothianidin; current response tests with α-bungarotoxin and thiacloprid

- Cfα3/Ggβ2: current response tests with acetylcholine, epibatidine, imidacloprid, nitenpyram, acetamiprid, dinotefuran and clothianidin
- Dmα1/Ggβ2: EC_{50} values, Hill coefficients and I_{max} values for acetylcholine, imidacloprid, nitenpyram, acetamiprid, dinotefuran and clothianidin; current response tests with α-bungarotoxin and thiacloprid
- Dmα2/Ggβ2: EC_{50} values, Hill coefficients and I_{max} values for epibatidine, I_{max} values for imidacloprid, nitenpyram, acetamiprid, dinotefuran and clothianidin; current response tests with α-bungarotoxin and thiacloprid
- Dmα3/Ggβ2: current response tests with acetylcholine, epibatidine, imidacloprid, nitenpyram, acetamiprid, dinotefuran and clothianidin
- Lcα1/Ggβ2: EC_{50} values, Hill coefficients and I_{max} values for acetylcholine, nicotine, imidacloprid, acetyl-*N*-ethylcholine, acetyl-*N*-propylcholine, acetyl-β-methylcholine, acetyl-α-methylcholine, trimethyl-(4-oxopentyl) ammonium, trimethyl-ethoxyethyl ammonium, trimethyl-(pentyl) ammonium, acetyl-thiocholine, carbamoylcholine, acetylhomocholine, choline and trimethyl-(3-methoxy-3-oxopropyl) ammonium; current response tests with α-bungarotoxin and phosphocholine
- Lcα2/Ggβ2: EC_{50} values, Hill coefficients and I_{max} values for acetylcholine, nicotine, epibatidine, imidacloprid, acetyl-*N*-ethylcholine, acetyl-*N*-propylcholine, acetyl-β-methylcholine, acetyl-α-methylcholine, trimethyl-(4-oxopentyl) ammonium, trimethyl-ethoxyethyl ammonium, trimethyl-(pentyl) ammonium, acetyl-thiocholine, carbamoylcholine, acetylhomocholine, choline and trimethyl-(3-methoxy-3-oxopropyl) ammonium; current response tests with α-bungarotoxin and phosphocholine
- Ggα4/Ggβ2: EC_{50} values, Hill coefficients and I_{max} values for acetylcholine, nicotine, imidacloprid, nitenpyram, acetamiprid, dinotefuran, clothianidin, acetyl-*N*-ethylcholine, acetyl-*N*-propylcholine,

acetyl-β-methylcholine, acetyl-α-methylcholine, trimethyl-(4-oxopentyl) ammonium, trimethyl-ethoxyethyl ammonium, trimethyl-(pentyl) ammonium, acetyl-thiocholine, carbamoylcholine, acetylhomocholine, choline and trimethyl-(3-methoxy-3-oxopropyl) ammonium; current response test with phosphocholine

Voltage clamp experiments and data analysis which led to the EC_{50} values and Hill coefficients of Cfα2/Ggβ2 and Dmα2/Ggβ2 for acetylcholine, imidacloprid, nitenpyram, acetamiprid, dinotefuran and clothianidin, as well as identification and isolation of subunit genes α1, α2 and α3 from *Ctenocephalides felis* and *Drosophila melanogaster,* and α4 and β2 nAChR subunit genes from *Gallus gallus* were carried out by Margaret Werr*. Chemical synthesis of acetylcholine derivatives were performed by myself under the supervision of Dr. Michael Berger*. Trimethyl-ethoxyethyl ammonium bromide was synthesized by Dr. Thorsten Meyer*. ^{1}H-NMR quantifications were performed by Stefan Derschum* and Dr. Thorsten Meyer*.

*: at MSD Animal Health Innovation GmbH in Schwabenheim an der Selz, Germany

INTRODUCTION

Cys-loop superfamily of ligand gated ion channels

Nicotinic acetylcholine receptors (nAChRs) belong to the Cys-loop (cysteine-loop) superfamily of the ligand gated ion channels (LGICs)[1] which comprises receptors for acetylcholine (nAChR), GABA ($GABA_A$ and $GABA_C$), glycine and serotonin (5-HT3). The LGICs act predominantly in fast neurotransmission at synaptic clefts (Han and Nichols 1996). Binding of neurotransmitters to the postsynaptic ion channels after their release from the presynaptic membrane leads to channel opening, which, in turn, causes depolarization and excitation, and hyperpolarization and inhibition of excitation of the postsynaptic membranes in the case of cation and anion channels, respectively (Haga 1996).

The members of the Cys-loop superfamily all share homologous structural features due to their origin from a common evolutionary ancestor (Ortells and Lunt 1995, Tasneem et al. 2005). These receptors are pentamers of subunits, each of which has an N-terminal ligand-binding extracellular domain of mainly β-sheets, four transmembrane regions presumably in mixed α/β topology (Corringer et al. 2000), cytoplasmic domains containing α-helix (Unwin 2005), and a short extracellular C-terminus. Depending on the number of different types of subunits for every receptor, they form homo- and heteropentamers. A highly characteristic sequence motif of all members of the superfamily is the 15-residue Cys-loop signature sequence formed by a Cys-Cys disulfide bridge, a closed loop that is located between the ligand binding domain and the first transmembrane helix (Ortells and Lunt 1995, Sine and Engel 2006).

Functionally, inhibitory receptor ion channels selective for anions (receptors for GABA and glycine) and excitatory receptor ion channels selective for cations (nAChRs and serotonin receptors) can be distinguished within the Cys-loop superfamily. Their anion/cation selectivity appears to be determined by the amino

[1] database: http://www.ebi.ac.uk/compneur-srv/LGICdb/LGICdb.php

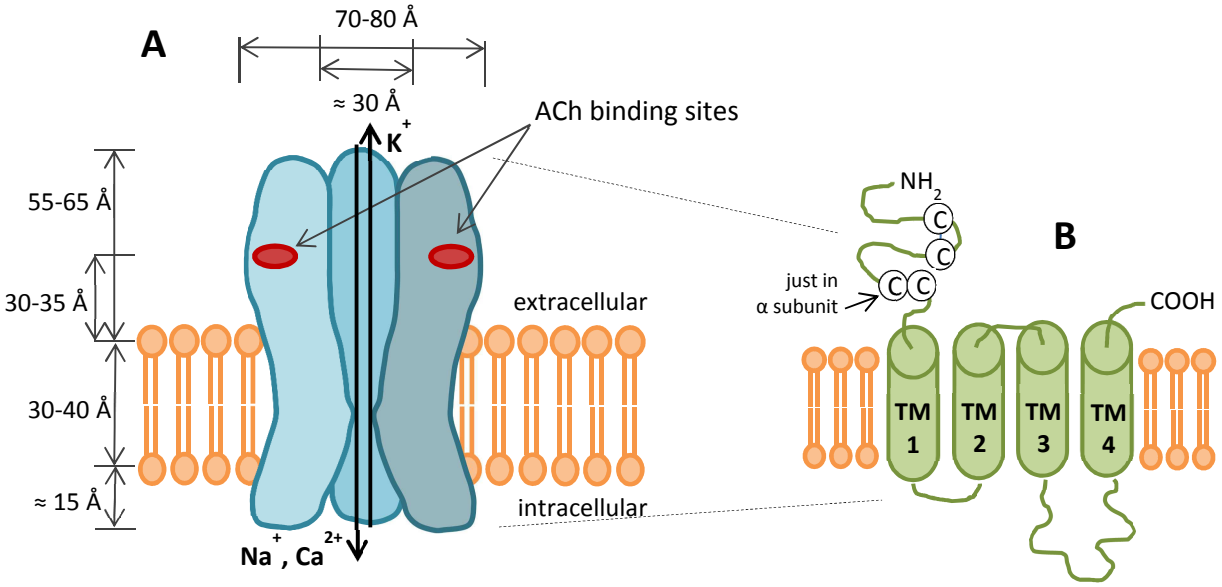


Figure 1: Schematic representation of a nicotinic acetylcholine receptor. A: Structural overview of a nAChR with two subunits removed revealing the channel tunnel. B: Structure of a nAChR subunit. (modified from Arias 2000, Matsuda et al. 2005)

acids lining the selectivity filter of the ion channel (Keramidas et al. 2004), which is mostly formed by the second transmembrane regions (TM2) of the five subunits. This organization of the selectivity filter is confirmed by mutational studies within or nearby the TM2 helices, which, depending on the exchanges, could cause the switch of ion selectivity from cations to anions (Galzi et al. 1992, Gunthorpe and Lummis 2001), and *vice versa* (Connolly and Wafford 2004, Keramidas et al. 2000). Due to the common evolutionary ancestor of the cation- and anion-conducting LGICs, there are several amino acids conserved, in some cases even across all members of the Cys-loop superfamily, in others within certain subtypes of the receptors. Most of these conserved amino acids contribute to ligand binding or ion gating (Le Novere and Changeux 2001, Sine and Engel 2006).

Although the narrowest point of the membrane pore, formed by amino acids near the intracellular end of TM2, determines ion flow through the receptor channel, the entire inner surface of the central vestibule (Fig. 1A) is a part of the selectivity filter. In the case of nAChR, the negatively charged groups lining their surface have the capacity to interact with cations and thereby may act stabilizing to their presence, which would increase the concentration of cation in the vestibule (Gunthorpe and Lummis 2001, Jensen et al. 2002, Keramidas et al. 2000, Unwin 2005, Wilson and Karlin 2001). The binding of the neurotransmitter leads to a conformation change in the ligand binding

domain which causes a reorientation of the TM2 helices resulting in their rotation and thus opening of the receptor channel (Miyazawa et al. 2003). In the case of the *Torpedo* electroplaque acetylcholine receptor, the narrowest point of the resting gate has a diameter of about 3Å, while through helix motion upon ACh binding the gate size increases to about 8Å (Unwin 1995).

Nicotinic Acetylcholine Receptor

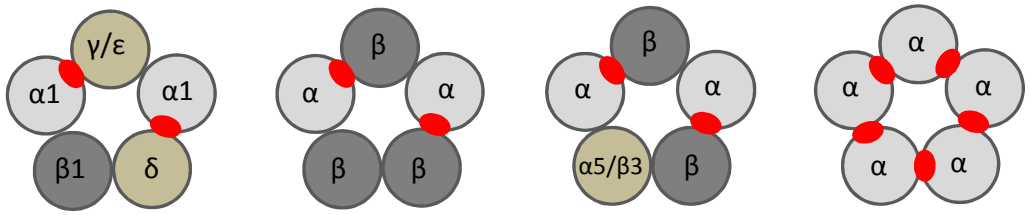
There are two groups of acetylcholine receptors (AChRs) differing in eponymous selective alkaloid agonists from natural sources: nicotinic AChR was named after nicotine from the tobacco plant (*Nicotiana tabacum*) whereas muscarinic AChR has its name from muscarine, a cholinergic drug first isolated from the mushroom fly agaric (*Amanita muscaria*) (Daly 2005). While nAChRs are ligand gated cation channels, muscarinic AChRs belong to the G-protein-coupled receptors.

The paradigmal nAChR from *Torpedo* is a glycoprotein of 290 kDa (Unwin 2005) consisting of five subunits, and it is thought that all nAChRs share this general structural setup. The quaternary structure of the pentamer is barrel-like and about 160 Å long (Arias 2000, Unwin 2005). The individual receptor subunits are of similar size (about 30 Å × 40 Å × 160 Å) and three-dimensional fold. In the nomenclature of the nAChR subunits, a distinction is drawn between α and non-α (β, γ, δ, ε) subunits: two adjacent cysteine residues, thought to be important for ACh binding, define nAChR α subunit, while non-α subunits lack this motif (Connolly and Wafford 2004, Le Novere and Changeux 1995, Le Novere et al. 2002, Ortells and Lunt 1995). To date, there is a large number of different subunits (10-30) identified in all vertebrate and invertebrate species. So potentially, many different subunit combinations are possible to form a heteropentameric receptor. However, not all subunits arrangements appear to be able to form functional ion channels and the number of proven pentamers is much lower than the theoretical number of potential combinations (Millar 2003). Nevertheless, the still considerable functional diversity of nAChRs is based on diversity of subunits (Le Novere et al. 2002, Millar 2003, Millar and Denholm 2007).

The nAChR is the best-investigated member of the Cys-loop superfamily and deemed to be its prototype. The state of knowledge of AChR has progressed in the last 40 years due to the availability of a source of abundant and highly enriched receptors: the electric organ of the electric ray, *Torpedo*. This specialized muscle-derived organ offers a high concentration of nAChRs which can be solubilized by detergents from the membrane with retained activity *in vitro* (Dolly and Barnard 1984, Grutter and Changeux 2001). An α-toxin from the venom of the snake *Bungarus*, α-bungarotoxin, proves to be highly selective for nAChRs and is shown to bind irreversibly to the *Torpedo* nAChR (Lee 1972, Lee and Chang 1966). This enables isolation and purification of nAChR from *Torpedo* by snake toxin affinity chromatography (Changeux et al. 1970, Corringer et al. 2000). Further, in the snail *Lymnaea stagnalis* a soluble ACh-binding protein (AChBP) was discovered (Smit et al. 2001) that is related to the N-terminal domain of the α-subunits of the nAChRs. Thus, the crystal structure of AChBP at 2.7Å resolution does predict structural properties of the nAChR ligand-binding domain, since almost all conserved nAChR residues are also present in this snail protein (Brejc et al. 2001). Recent electron microscopic experiments on tubular crystals from *Torpedo* postsynaptic membranes at 4 Å resolution (Unwin 2005), X-ray structures of extracellular domain of the mouse nAChR α1 subunit bound to α-bungarotoxin at 1.94 Å (Dellisanti et al. 2007), and of prokaryotic LGICs from the gram negative bacterium *Erwinia chrysanthemi* (Hilf and Dutzler 2008) and from the cyanobacterium *Gloebacter violaceus* (Bocquet et al. 2009, Hilf and Dutzler 2009) at 3.3 Å and 3.1 Å resolution, respectively, provide an insight into the architecture of nAChR and LGICs in general. At a functional level, the possibility of expressing correctly folded ion channels in *Xenopus laevis* oocytes permits structural ligand binding, electrophysiology and receptor regulation studies on nAChRs (Dascal 1987, Snutch 1988).

Vertebrate nAChRs

To date, seventeen different nAChR subunits have been identified in vertebrates (α1-α10, β1-β4, γ, δ, ε). Most nAChRs coassemble into heteropentameric receptors containing at least one type of α subunit and one type of non-α subunit, but some receptors are homopentamers formed by a single α subunit type (e.g. α7) (Millar and



muscle-type nAChRs
α1, β1, δ, γ, ε

heteromeric neuronal nAChRs
α2-α6, β2-β4

homomeric neuronal nAChRs
α7, α9

Figure 2: Various heteropentameric and homopentameric human nAChR subtypes (modified from Jensen et al. 2005). Red ovals: ACh binding sites.

Gotti 2009). The pentamers of vertebrate muscle-type nAChRs have a fixed stoichiometry of four differend subunits ($(\alpha1)_2\beta1\delta\varepsilon/\gamma$, Fig. 2) and are present postsynaptically at the neuromuscular junction. Neuronal nAChRs are located at presynaptic and postsynaptic membranes in the autonomic ganglia and the central nervous system (Jensen et al. 2005, Wonnacott 1997). The subunit stoichiometries of the neuronal representatives of nAChR subtypes are less clearly defined. In general, the most heteromeric neuronal nAChRs seem to be coassemblies of two α subunits (one or two types) and three β subunits (one or two types) (Millar 2003, Millar and Gotti 2009). Neuronal nAChR expression is tissue-specific and the predominant subunits in the central nervous system have been identified as α7, α4 and β2. By contrast, in the autonomic ganglia, abundant expression of α3 and β4 is noted (Jensen et al. 2005, Paterson and Nordberg 2000). The difficulty of investigating subunit combinations arises also out of discrepancies in results obtained from recombinant *versus* native nAChR (Millar and Gotti 2009).

The diversity of nAChR subunit compositions gives rise to different physiological and pharmacological receptor properties. In humans, nAChRs represent drug targets in the pathology of several neurological and neuromuscular disorders. These include neuropathic pain, Alzheimer's and Parkinson's diseases, myasthenia gravis, congenital myasthenic syndromes, schizophrenia, depression, attention deficit disorders, tobacco addiction, epilepsy and Tourette's syndrome (D'hoedt and Bertrand 2009, Jensen et al. 2005, Lindstrom 2000, 2003, Livett et al. 2006, Paterson and Nordberg 2000, Steinlein 2007, Taly et al. 2009, Weiland et al. 2000). Interestingly, some partial agonists and antagonists addressing specific nAChR subtypes as well as cholinesterase inhibitors show efficacy in the treatment of alcohol

abuse and dependence (Rahman and Prendergast 2012). There is evidence of deleterious, but also beneficial connections between nicotine exposure and several diseases. Smokers have a lower prevalence of Alzheimer's and Parkinson's diseases (Brenner et al. 1993, Quik et al. 2009), whereas depression, attention deficit disorders and schizophrenia are over-represented among smokers. Thereby it is assumed that smoking may occasionally serve as a kind of self-medication (Glassman et al. 1990, Lohr and Flynn 1992, Mineur and Picciotto 2010, Pomerleau et al. 1995). In fact, there is evidence that nicotine consumption has antidepressant property (Salin-Pascual et al. 1995), can improve attention and memory, and significantly decreases attention deficit disorder symptoms (Levin and Simon 1998). In addition to the nervous system, many components of the cholinergic system are found also in non-neuronal cells (Sharma and Vijayaraghavan 2002, Wessler and Kirkpatrick 2008). There is evidence of nAChRs being involved in regulation of cell proliferation, in apoptosis, migration, invasion, angiogenesis and in secretion (Egleton et al. 2008). Nicotine is found to upregulate the nAChRs during continuing exposition (Vallejo et al. 2005), and to stimulate tumor growth and survival of non-neuronal cells (Egleton et al. 2008). Further, nAChRs expressed in lymphocytes are thought to be involved in inflammation regulation (Borovikova et al. 2000, Wessler and Kirkpatrick 2008) and modulation of immune system function (Fujii et al. 2012, Kawashima and Fujii 2000) like T-cell activation (Egleton et al. 2009) or regulation of antibody production (Kawashima et al. 2007).

Insect nAChRs

In contrast to their vertebrate counterparts, insect nAChRs are less well explored. To date, they are the smallest nAChRs gene families known (Jones et al. 2007) with e.g. 10 genes in *Drosophila (D.) melanogaster* (Dmα1-Dmα7, Dmβ1-Dmβ3, (Littleton and Ganetzky 2000, Sattelle et al. 2005)) and *Anopheles gambiae* (Agα1-Agα9, Agβ1, (Jones et al. 2005a)), 11 genes in *Apis mellifera* (Amα1-Amα9, Amβ1-Amβ2, (Jones et al. 2006)), and 12 genes in *Bombyx mori* (Bmα1-Bmα9, Bmβ1-Bmβ3, (Shao et al. 2007)). Other insect nAChR subunit genes have been cloned from e.g. planthopper *Nilaparvata lugens* (Liu et al. 2006), green peach aphid *Myzus persicae* (Huang et al. 1999), asiatic honey bee *Apis cerana cerana* (Yu et al. 2011), desert locust

Schistocerca gregaria (Jones et al. 2005b, Marshall et al. 1990), and nicotine-insensitive tobacco hornworm *Manduca sexta* (Eastham et al. 1998).

Based on sequence homology analysis, the nAChR gene families of *D. melanogaster*, *Anopheles gambiae* and *Apis mellifera* possess groups of subunits that are highly conserved between these species (with >60% sequence identity). However, each insect gene family also have at least one divergent subunit with low sequence homology (with <20% sequence homology), which could stand for species-specific receptor subtypes (Jones et al. 2007). Furthermore, some insect nAChR subunits are known to undergo alternative splicing and RNA editing (Jones et al. 2005a, 2006, Lansdell and Millar 2000a, Sattelle et al. 2005), what also could generate species-specific receptor subtypes. But presently, knowledge about insect nAChR subtype diversity in the nervous system is still fragmentary (Tomizawa and Casida 2001).

In vertebrates, the neuromuscular transmission is cholinergic and the vast majority of excitatory neurotransmission in the brain are mediated by glutamate (Dingledine et al. 1999). This is fundamentally different in insects, where the main excitatory neurotransmitter in the brain is acetylcholine, while the synaptic transmission at the neuromuscular junction is glutamatergic (Millar and Denholm 2007, Sattelle 1980). Due to the fact that the insect nervous system is one of the richest sources of nAChRs (Breer and Sattelle 1987, Dudai 1978), it provides the opportunity to exploit these receptor ion channels as selective targets for nerve poisoning insecticides (Jeschke 2007, Matsuda et al. 2001). Recent surveys report that by market share, about 90% of synthetic insecticides are neurotoxins acting on only four targets: acetylcholinesterases, the voltage-dependent sodium channels, the GABA-gated chloride channels, and nAChRs (Tomizawa and Casida 2003). Insecticides binding to nAChRs have a market share in agriculture of ~24% (Jeschke et al. 2011), and the by far largest and commercially most important insecticide class addressing these receptors are the neonicotinoids with the forerunner imidacloprid, which is marketed since 1991 (Jeschke and Nauen 2008, Millar and Denholm 2007). To date, imidacloprid is the major selling insecticide in the world and holds over 40% of the whole neonicotinoid market (Jeschke et al. 2011).

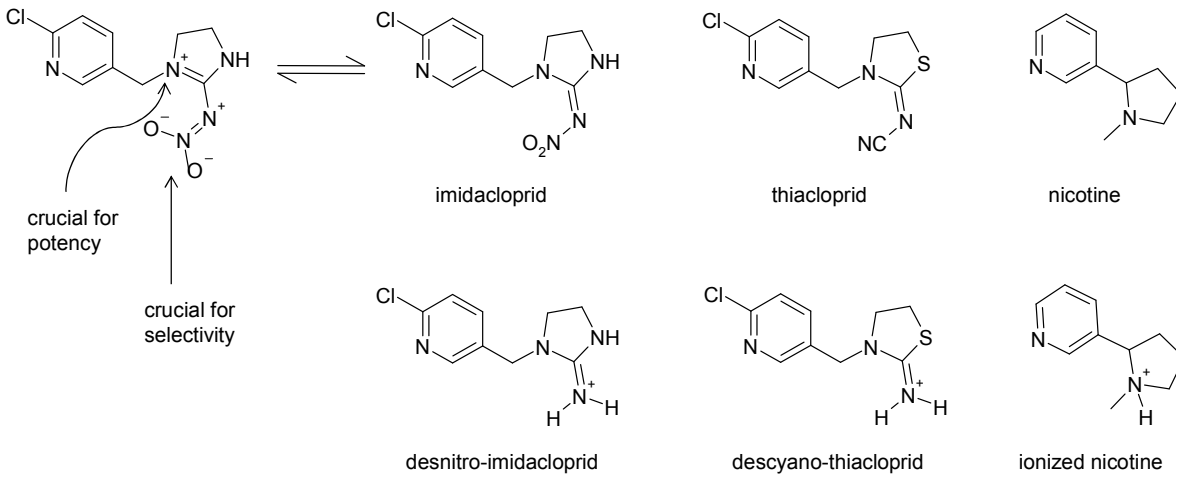


Figure 3: Structures of imidacloprid and thiacloprid, their desnitro/descyano-counterparts, mesomeric structure of imidacloprid, and nicotine in ionized and nonionized form.

Nicotine is a naturally occurring insecticide (Soloway 1976), but its commercial use is limited due to its high toxicity to vertebrates, which is higher than its insecticidal potency (Millar and Denholm 2007). In contrast, neonicotinoids, with their structural similarity to nicotine and a common mode of action (Tomizawa and Casida 2003) are more than 100-fold selective for insect over vertebrate nAChRs. Yamamoto et al. (1998) points out the significance of compound hydrophobicity in selective action of insecticides. The amino group nitrogen atom of nicotinoids is ionized under physiological conditions. This ionisation is essential for interaction with vertebrate nAChRs but results in poor penetration of the ion-impermeable barrier of insect CNS (Tomizawa and Casida 2003, Yamamoto et al. 1998). In contrast, neonicotinoids are not protonated at physiological pH (Fig. 3), which allows for more efficient penetration into the insect CNS, while the formation of zwitterionic mesomeric structures ensure potency on insect nAChRs as well as selectivity over vertebrate receptors (Tomizawa and Casida 2003, Tomizawa et al. 2000). Remarkably, desnitro-imidacloprid and descyano-thiacloprid are considerably more potent on vertebrate than on insect nAChRs (Matsuda et al. 2001, Tomizawa and Casida 2003, Tomizawa et al. 2000), indicating the nitro or cyano substituents enabling the mesomeric structures are crucial for insect *versus* vertebrate nAChRs selectivity (so-called “magic” nitro and “magic” cyano groups) (Tomizawa and Casida 2003). This could be explained by assumption of the architecture differences in insect *versus* vertebrate nAChR ligand binding sites: the negatively charged nitro/cyano groups interact with cationic amino acid residue(s) in insect receptors, while in the case of the positively charged

moieties in desnitro/descyano compounds, interaction with insect nAChRs is weak, but strong with anionic subside mammalian nAChRs (Tomizawa and Casida 2005, Tomizawa and Casida 2011, Tomizawa et al. 2000).

The molecular definition of the insecticide target site in insect nAChRs is still incomplete due to the difficulties in the expression of recombinant receptor channels in heterologous expression systems (Millar 1999, Millar and Lansdell 2010). Except for the homomeric *Schistocerca* nAChR α1 expressed in *Xenopus* oocytes (Amar et al. 1995, Marshall et al. 1990), functional expression of other insect nAChRs in *Xenopus* oocytes is limited to trans-species hybrid receptor formation of insect α subunits with chicken or rat β2 subunits (Bertrand et al. 1994, Millar 1999, 2009, Millar and Lansdell 2010, Tomizawa et al. 2005). Similarly, expression of insect nAChRs in cultured mammalian as well as insect cell lines is also only possible by coexpression with vertebrate β2 subunits (Huang et al. 1999, Lansdell and Millar 2000b, Lansdell et al. 1997, Millar 2009). Thus, exploring pharmacological profiles of insect nAChRs in the currently avaliable recombinant settings clearly does not reflect the *in vivo* situation and requires stringent controls and cautious interpretation.

Architecture of the ACh binding site

One way to obtain molecular insights in the nAChR binding site is the analysis of crystal structures. While the generation of X-ray structures of the entire nAChR channel has so far not been successful, crystallization of mollusk AChBPs has been achieved. Although lacking the ion channel domain, based on homology considerations, AChBP is thought to be equivalent to the nAChR binding domain and contains many of the residues thought to be crucial for tertiary structure and ligand binding (Brejc et al. 2001). In concordance with this view, AChBP binds known nAChR agonists and competitive antagonists (Brejc et al. 2001, Smit et al. 2001). Crystal structures of *Lymnaea stagnalis (Ls)* AChBP in complex with imidacloprid and clothianidin (Ihara et al. 2008), and with nicotine and carbamylcholine (Celie et al. 2004), respectively, and of *Aplysia californica (Ac)* AChBP in complex with thiacloprid and imidacloprid (Talley et al. 2008) allow first conclusions about the interactions of agonists with amino acid residues of the binding pocket. Further X-ray studies with neonicotinoids and nicotinoids interacting with these two AChBPs show that *Ac*-

AChBP exhibits high affinity for neonicotinoids, while *Ls*-AChBP has low neonicotinoid and high nicotinoid binding affinity. Thus, it is proposed that *Ac*-AChBP could serve as a surrogate for insect nAChRs and *Ls*-AChBP for vertebrate nAChRs (Tomizawa and Casida 2009, Tomizawa and Casida 2011).

The ACh binding site is located at the interface between two subunits and consists of two components: the so-called “principal” and “complementary” sides (Corringer et al. 1995). The principal component is on the α subunit and built up of the three loops A-C. The three further peptide loops D-F are part of the complementary side located on the non-α subunit in heteropentamers, and on the neighbouring α subunit in homopentamers like α7, respectively (Corringer et al. 2000, Karlin 2002). Thus, heteromeric and homopentameric nAChRs have typically two and five ACh binding sites, respectively (Fig. 2).

Some functionally significant amino acids of the six loops involved in ACh binding are conserved across the nAChRs (Brejc et al. 2001, Corringer et al. 2000, Jensen et al. 2005, Sine and Engel 2006). Thus, the variable amino acids are likely to contribute to the subunit and species selectivity, as well as to the distinct pharmacological profiles of different nAChRs (Corringer et al. 1998, Grutter and Changeux 2001, Tomizawa and Casida 2009, Tomizawa and Casida 2011). Among the conserved amino acids, there are two adjacent cysteine residues located at the turn of β hairpin folded loop C (Brejc et al. 2001) and enclosed by two tyrosines (Abramson et al. 1989, Dennis et al. 1988). These two aromatic residues build together with tyrosine from loop A (Galzi et al. 1990) and two tryptophans from loops B (Dennis et al. 1988) and D (Chiara and Cohen 1997) the so-called “aromatic box” (Fig. 4), which forms the binding site for the positively charged quaternary ammonium group of ACh (Jensen et al. 2005), probably *via* forming cation-π interaction with a tryptophan of loop B (Dougherty and Stauffer 1990, Jensen et al. 2005, Zhong et al. 1998). Zhong et al. (1998) found that a decrease in cation-π binding ability for various tryptophan derivatives at this defined position in loop B increases the EC_{50} (effective agonist concentration for 50% maximum response) values for ACh. In the ligand-free form of the receptor, loop C projects away from the body of the α subunit, whereas in ligand-bound conformation loop C is closer to loops A and B surrounding the ligand, burying them and tightly capping the binding pocket (Tomizawa and Casida 2009, Unwin 2005). Furthermore, the ligand seems to form contacts more with the principal side of the binding pocket

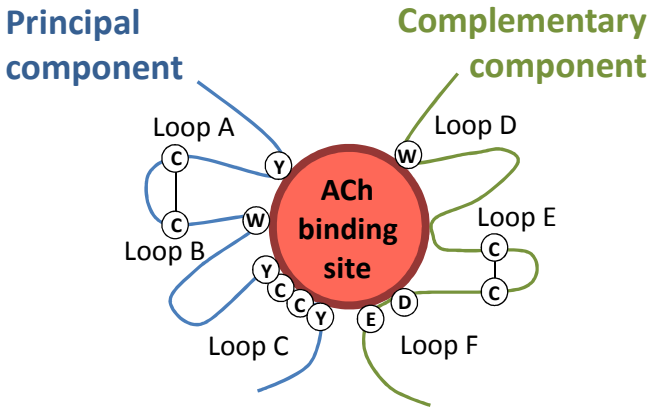


Figure 4: Ligand binding site of the nAChRs (modified from Matsuda et al. 2005)

rather than with the complementary side, an arrangement known for nicotine and carbamylcholine bound to AChBP (Celie et al. 2004). Shimomura et al. (2004) found that the single amino acid between the tyrosine and the two adjacent cysteins in loop C as well as the loop B-C interval length differ considerably between insect and vertebrate α subunits, and these differences have an influence on neonicotinoid sensitivity (Matsuda et al. 2005). Loop D includes basic residues, which can contribute to the binding stability of neonicotinoids in insect nAChRs (Matsuda et al. 2009). In agreement with these findings, mutations in loop D of chicken α7 and α4/β2 receptors to corresponding basic residues result in significant enhancement of neonicotinoid sensitivity (Shimomura et al. 2002, 2006, Toshima et al. 2009). Further mutation studies highlighted the possible role of loop E as a contributor to neonicotinoid binding efficiency (Amiri et al. 2008).

Pharmacological profiles of native nAChRs show that - similar to the findings with mammalian nAChRs - α-bungarotoxin-sensitive and -insensitive nAChR forms exist in insects (Thany et al. 2007). Based upon studies with *D. melanogaster* hybrid receptors, Dmα1- and Dmα3-containing nAChRs are sensitive to α-bungarotoxin, while Dmα2- and Dmα4-containing hybrid receptors are insensitive (Bertrand et al. 1994, Lansdell et al. 2000a,b, Schulz et al. 1998, Tomizawa et al. 2005). Some other naturally occurring toxins bind to different regions of the binding pocket, as is shown for the cone snail peptide α-conotoxin ImI, the plant alkaloid methyllycaconitine and the tree frog alkaloid epibatidine (Taylor et al. 2007). Since nitenpyram resistant mutants of *D. melanogaster* are also cross-resistant to imidacloprid and

thiamethoxam (Perry et al. 2008), neonicotinoids appear to bind to an equivalent region. But the neonicotinoid binding site seems to overlap only partially with that of α-bungarotoxin (Nishiwaki et al. 2003, Tomizawa et al. 2005) or epibatidine (Tomizawa et al. 2005).

An additional way of studing features of the binding site in nAChRs is structure-activity data analysis. Schmitt (2000) classified in his review an impressive data set over non-peptidic ligand structure-affinity relationships on CNS nAChRs α4/β2 and α7. The compounds were grouped into five classes based on structures of their cationic centers and hydrogen bond acceptor/π moieties – the two crucial features for activity on nAChRs (Schmitt 2000). For insect nAChRs ligands, similar studies have not yet been reported.

While particularly the last decade has seen a tremendous increase in nAChR structural knowledge, there are many details that are still not known definitely about interaction between ligand and binding site of nAChRs. So until the *in vivo* subunit compositions, as well as the full crystal structure of defined nAChRs are established, structure-activity data interpretation will remain limited.

AIM OF THE STUDY

Part I: Pharmacological characterisation of nAChR α subunits of the cat flea Ctenocephalides felis

By market share, nAChR is one of the four insecticidal targets of capital importance (Tomizawa and Casida 2003). However, nAChR gene sequences are only known for a limited number of pest insects. Prevention and treatment of ectoparasite infestation of companion animals, such as cats and dogs, is a major market in animal health. The International Federation for Animal Health estimated the count of the world cat population to be about 220 million (in 2010, IFAH 2012). Of the many parasites of cats, the cat flea *Ctenocephalides (C.) felis* is a major source of discomfort and a transmitter of diseases (Rust 2005). Some of established cat flea insecticides are neonicotinoids such as imidacloprid, nitenpyram and dinotefuran (Rust 2005), which act on nAChRs.

At the beginning of this study, only a single report on nAChRs of *C. felis* had been published, reporting the partial gene cloning of eight nAChR subunits Cfα1 – Cfα4, Cfα7, Cfα8 and Cfβ1, and providing limited functional studies by radioligand binding experiments (Bass et al. 2006). The initial task of the first part was to identify and clone the full length cDNA sequences of the Cfα1, Cfα2 and Cfα3 genes based on our own degenerate primer RT-PCR data (not shown) and on the published partial cDNA sequences DQ237865, DQ237866 and DQ237867 (Bass et al. 2006). The second task was the functional expression of the new subunits in *Xenopus* oocytes as trans-species heteromers with chicken β2 (Ggβ2) subunit. Furthermore, these three hybrid receptors (Cfα1/Ggβ2, Cfα2/Ggβ2 and Cfα3/Ggβ2) were pharmacologically characterized in two electrode voltage clamp experiments with the natural ligand ACh, the standard agonists nicotine and epibatidine, and the paradigmatic inhibitor α-bungarotoxin. In the fourth task, the pharmacology of the neonicotinoid market products imidacloprid, nitenpyram, acetamiprid, thiacloprid, dinotefuran and clothianidin (Fig. 5) was determined for the *C. felis* subunits Cfα1 and Cfα2, and compared with those of the chicken α4 subunit, in their respective chicken β2 subunit heteromer context (Cfα1/Ggβ2, Cfα2/Ggβ2, Ggα4/Ggβ2).

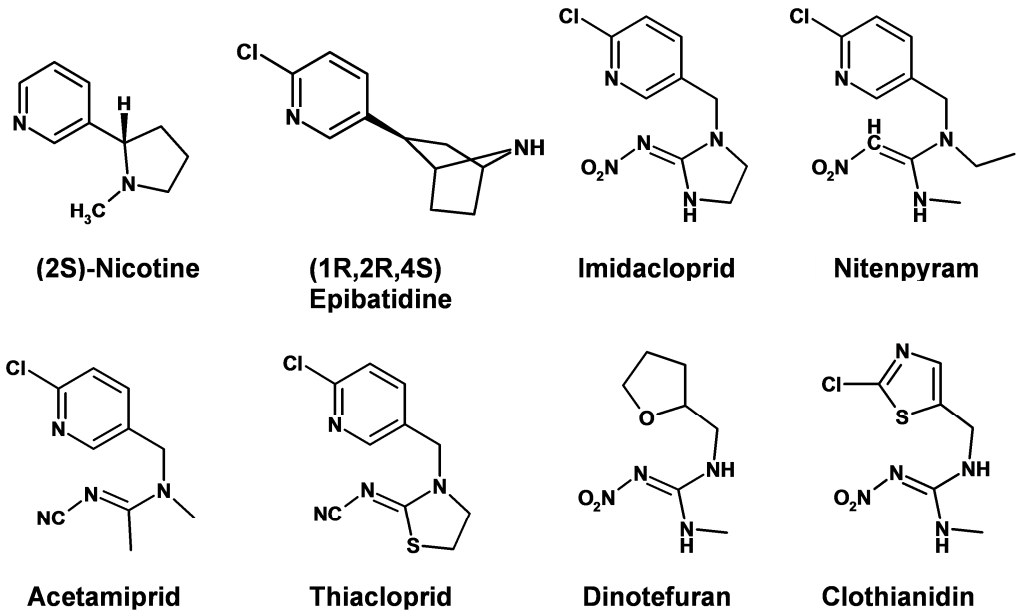


Figure 5: Chemical structures of nicotinoids and neonicotinoids applied in this study. From Dederer et al. 2011.

Additionally, in the fifth task, the pharmacology of these *C. felis* subunits was compared with the respective subunits of the insect model organism *D. melanogaster.*

Part II: Molecular cloning and functional expression of Lucilia cuprina *α subunits – pharmacology and study of structure-activity relationships using chemically synthesized acetylcholine derivatives*

The sheep blowfly *Lucilia* (*L.) cuprina* is the causative agent of major animal distress and economic losses in sheep husbandry in subtropical areas. Spinosad is an established blowfly insecticide (Levot et al. 2002), known to act on receptors containing nAChR subunit α6 (Baxter et al. 2010, Perry et al. 2007). So far, no compound of the neonicotinoid class has been registered as a market product against *L. cuprina*. In bioscreen experiments in MSD Animal Health Innovation GmbH, imidacloprid and nitenpyram showed some killing efficacy on *L. cuprina* larvae (Dr. H. Williams, unpublished observations). This prompted our interest to

investigate the nAChR subunits of this important insect parasite as potential pesticide targets.

In part II of this study the following subprojects were pursued: first, the genes of two *L. cuprina* nAChR subunits Lcα1 and Lcα2 were identified and their full length cDNAs cloned. In the second task, these two new subunits were functionally expressed in *Xenopus laevis* oocytes as trans-species heteromers with the chicken nAChR β2 subunit. The third task was – analogous to part I – the pharmacological characterisation of the two *L. cuprina* heterologous nAChRs with ACh, the nicotinoids nicotine and epibatidine, the antagonist α-bungarotoxin, and, for comparison, with the neonicotinoid imidacloprid. The fourth task comprised the chemical synthesis of eight close ACh derivatives and their characterisation together with an additional five purchased ACh analoga (for structures see Figure 6, p. 32). In the last task, structure-activity relationships for agonist action of these compounds were established and compared for Lcα1/Ggβ2, Lcα2/Ggβ2 and Ggα4/Ggβ2 receptors. The results were expected to give guidance in the identification of fly-specific compounds.

RESULTS AND DISCUSSION

This study is the first report about *de novo* full length identification and cloning of three *C. felis* nicotinic acetylcholine receptor genes α1, α2 and α3 (*cfα1*, *cfα2*, *cfα3*), as well as two *L. cuprina* genes α1 and α2 (*lcα1* and *lcα2*). The deduced polypeptides from these sequences contain motifs typical for nicotinic receptor α subunit ligand binding sites within the putative extracellular domains (Albuquerque et al. 2009, Arias 1997, Corringer et al. 2000): the cysteine loop and the loops A-C with a number of highly conserved amino acid residues. Furthermore, four transmembrane helices typical for nAChR subunits and LGICs in general (Albuquerque et al. 2009, Arias 1997, Corringer et al. 2000) are predicted in the polypeptide sequence analysis of all five subunits. Finally, every subunit contains the highly conserved amino acid residues of the nAChR ion channel 'rings' within and neighbouring to the second transmembrane helix TM2.

To examine functionality and properties of the full length nAChR cDNAs identified in this study, they were coexpressed in *Xenopus laevis* oocytes with the chicken β2 (Ggβ2) subunit (Bertrand et al. 1994) and studied by two-electrode voltage clamp assays. In all cases, the electrophysiological functionality of the α subunits was shown by application of ACh, which led to dose-dependent currents. These currents were rather weak in the case of Cfα3/Ggβ2 and Lcα1/Ggβ2 (largely nA range), but strong in the case of Cfα1/Ggβ2, Cfα2/Ggβ2 and Lcα2/Ggβ2 (up to 30μA). In the case of Cfα2/Ggβ2 and Lcα2/Ggβ2, the EC_{50} values for ACh were 9.0 μM and 5.37 μM, respectively. In contrast, Cfα1/Ggβ2 and Lcα1/Ggβ2 were much more sensitive to ACh activation with EC_{50} values of 50 nM and 80 nM, respectively. For Cfα3/Ggβ2, an EC_{50} for ACh could not be determined in *Xenopus* two electrode voltage clamp experiments, due to the low signals, which also precluded detailed analysis of other agonists used in this study. A similar picture was seen for the corresponding *D. melanogaster* orthologs: for Dmα2/Ggβ2, strong ACh-elicited currents and an EC_{50} of 6.6 μM were detected, while the combination Dmα1/Ggβ2 proved to be much more sensitive than the Dmα2 combination, with an EC_{50} of 70 nM. Like Cfα3/Ggβ2, Dmα3/Ggβ2 also gave only small ACh-dependent currents, precluding EC_{50}

determination for this ligand and other agonists used in this study. Cfα2/Ggβ2 and Lcα2/Ggβ2 were insensitive to α-bungarotoxin at 2 μM, while ACh-induced currents of the Cfα1/Ggβ2 and Lcα1/Ggβ2 combinations were completely blocked by this concentration of snake toxin. These characteristics of α-bungarotoxin response have been postulated as hallmarks of insect α1 and α2 subunits (Bertrand et al. 1994, Lansdell and Millar 2000b), a conclusion confirmed in this study.

Further, the four newly identified α1 and α2 subunits were tested upon their nicotine, epibatidine and imidacloprid sensitivity. In the case of both α1 hybrid subunit combinations, as well as the comparative Dmα1/Ggβ2, even low epibatidine concentrations led to a persistent activation of the receptors, which precluded the determination of proper dose response curves. Repeated epibatidine application resulted in pronounced desensitization of Lcα2/Ggβ2, while nicotine desensitized both Lcα1/Ggβ2 and Lcα2/Ggβ2. As these effects could not be reversed by washing, the calculated EC_{50} and I_{max} values have to remain tentative, and Hill coefficients were not determined. The neonicotinoid imidacloprid gave rise to only small currents on Lcα1/Ggβ2 that made dose-response considerations difficult and led only to tentative EC_{50} values. The sensitivity of Cfα1/Ggβ2 and Lcα1/Ggβ2 combinations to agonists in general was one or two orders of magnitude higher compared to Cfα2/Ggβ2 and Lcα2/Ggβ2. The (neo)nicotinoid agonist potencies for both α2 hybrid subunit combinations Cfα2/Ggβ2 and Lcα2/Ggβ2 decreased in the following order: epibatidine > imidacloprid > nicotine. The EC_{50} values for imidacloprid and nicotine on the both α1 hybrid subunit combinations were all approximately 20 nM. The *D. melanogaster* orthologs Dmα1 and Dmα2 in combinations with Ggβ2 showed a similar picture concerning EC_{50} values and orders of potency as the four new α subunits – a finding different from earlier reports (Ihara et al. 2003). The nicotinoid and neonicotinoid activity data obtained from the three insect receptor subunit studies were compared with those from the chicken neuronal receptor Ggα4/Ggβ2 also expressed in *Xenopus* oocytes. Epibatidine treatment led to persistent activation of Ggα4/Ggβ2 that could only insufficiently be washed off, which precluded EC_{50} determinations. Imidacloprid showed lower potency on Ggα4/Ggβ2 than nicotine, with EC_{50} values of 13.8 μM and 760 nM, respectively. Thus, imidacloprid was 690-fold less potent on the chicken receptor Ggα4/Ggβ2 than on Cfα1/Ggβ2 and

Lcα1/Ggβ2, and 11-fold and 22-fold less potent than on Cfα2/Ggβ2 and Lcα2/Ggβ2, respectively.

In addition, both cat flea nAChR α1 and α2 subunits, as well as, for comparison, Dmα1/Ggβ2, Dmα2/Ggβ2 and Ggα4/Ggβ2, were tested for their sensitivity to further neonicotinoids derivatives. The agonist potencies of all nicotinoids and neonicotinoids tested on Cfα2/Ggβ2 increased in the following order: dinotefuran < nitenpyram < nicotine ~ acetamiprid < clothianidin < imidacloprid < epibatidine, while on Cfα1/Ggβ2 the order was dinotefuran < nitenpyram < clothianidin < acetamiprid < nicotine < imidacloprid. While the EC_{50} of imidacloprid (1.31 μM) on the potential neonicotinoid target Cfα2/Ggβ2 was in an activity range that could perhaps be in agreement with its potent anti-flea activity, the potency of the other flea control market products nitenpyram (EC_{50} = 24.4 μM) or dinotefuran (EC_{50} = 124.8 μM) appeared too low to explain their *in vivo* activity based on interaction with this insect receptor subunit. The sensitivity of the Cfα1/Ggβ2 hybrid subunit combination to neonicotinoid ligands was about 10-100 times higher compared to Cfα2/Ggβ2 with EC_{50} values mostly in the nanomolar range, except for dinotefuran (EC_{50} = 4.19 μM). Further, I_{max} determinations with ACh as standard showed that all nicotinoid and neonicotinoid ligands investigated here were partial agonists on Cfα1/Ggβ2. On Cfα2/Ggβ2, clothianidin behaved as a superagonist, dinotefuran and epibatidine as full agonists, while all the other ligands were partial agonists. Thiacloprid was also an agonist of Cfα2/Ggβ2 and Cfα1/Ggβ2, but EC_{50} or I_{max} values could not be determined, because in contrast to other neonicotinoids, repeated application of this derivative led to a gradual loss of signal, even at low concentrations. Replacement of Cfα1 and Cfα2 by their *D. melanogaster* orthologs Dmα1 and Dmα2 in *Xenopus* expression and electrophysiology experiments with the neonicotinoid agonists nitenpyram, acetamiprid, clothianidin and dinotefuran revealed similar EC_{50} values and orders of potency as for their *C. felis* counterparts. Dose-response investigations of neonicotinoids on Ggα4/Ggβ2 in *Xenopus* oocytes showed that all 5 neonicotinoid compounds investigated were partial agonists in the μM to mM range. EC_{50} determinations showed that nitenpyram was 73-fold, acetamiprid 85-fold, dinotefuran 244-fold and clothianidin 142-fold less potent on the chicken receptor Ggα4/Ggβ2

than on Cfα1/Ggβ2. These selectivities may provide a potential rational definition of structure-activity and structure-selectivity relationships for insecticide design.

The electrophysiology comparisons of the *C. felis, L. cuprina* and *D. melanogaster* α subunits in this study suggest that the insect α1 subunit may be a better candidate for being a crucial part of the *in vivo* neonicotinoid target than insect α2, which has been in the center of many previous investigations. This notion is supported by a number of earlier studies. Liu et al. (2009) showed differences in EC_{50} values for ACh and imidacloprid on *Nilaparvata (N.) lugens* nAChR subunits Nlα2 or Nlα1 coexpressed with the rat β2 subunit in *Xenopus* oocytes. Whereas ACh was twofold more potent on Nlα1/Rnβ2 than on Nlα2/Rnβ2, imidacloprid shows 15-fold more potency (Liu et al. 2009). Further, in *N. lugens* as well as in *D. melanogaster,* target site-based neonicotinoid resistance has been reported. In *N. lugens*, $Y^{151}S$ mutations have been identified in the nAChR subunits α1 and α3 that have been implicated in neonicotinoid resistance (Li et al. 2010, Liu et al. 2005, 2006, 2009, Yixi et al. 2009). Mutagenesis studies in *D. melanogaster* led to the isolation of two neonicotinoid-resistant strains that had lesions in the Dmα1 and the Dmβ2 genes, respectively (Perry et al. 2008). The combined data of our current and earlier studies suggest that insect α1 may play a central role in neonicotinoid insecticide action.

In order to obtain a more detailed picture about the structural requirements for agonist action on Lcα1 and Lcα2-containing nAChRs, a collection of 13 close derivatives of the natural ligand ACh was assembled (Fig. 6). ACh and five derivatives (**1**, **4**, **9**, **10**, **12**, **13**) could be purchased, while eight further compounds (**2**, **3**, **5**, **6**, **7**, **8**, **11**, **14**) were synthesized in this study. Five centres for derivatization of ACh were chosen: first, one methyl group (R^1) of the quaternary ammonium group was replaced by ethyl (**2**) and n-propyl (**3**). The ethylene group was substituted by methyl either in the α-(R^2) or in the β-position (R^3) yielding *rac*-α-methylcholine (**5**) and *rac*-β-methylcholine (**4**), respectively. The choline oxygen (X) experienced the most modifications: replacement of the oxygen by sulphur led to acetylthiocholine (**9**), while replacement by a methylene group yielded trimethyl-(4-oxopentyl)-ammonium (**6**). Homologation of the ethylene group by one carbon led to acetylhomocholine (**11**), while replacement of the acetyl group by -H or by $-PO_3^{2-}$ led to choline (**12**) and phosphocholine (**13**), respectively. Inversion of the ester function in ACh led to the

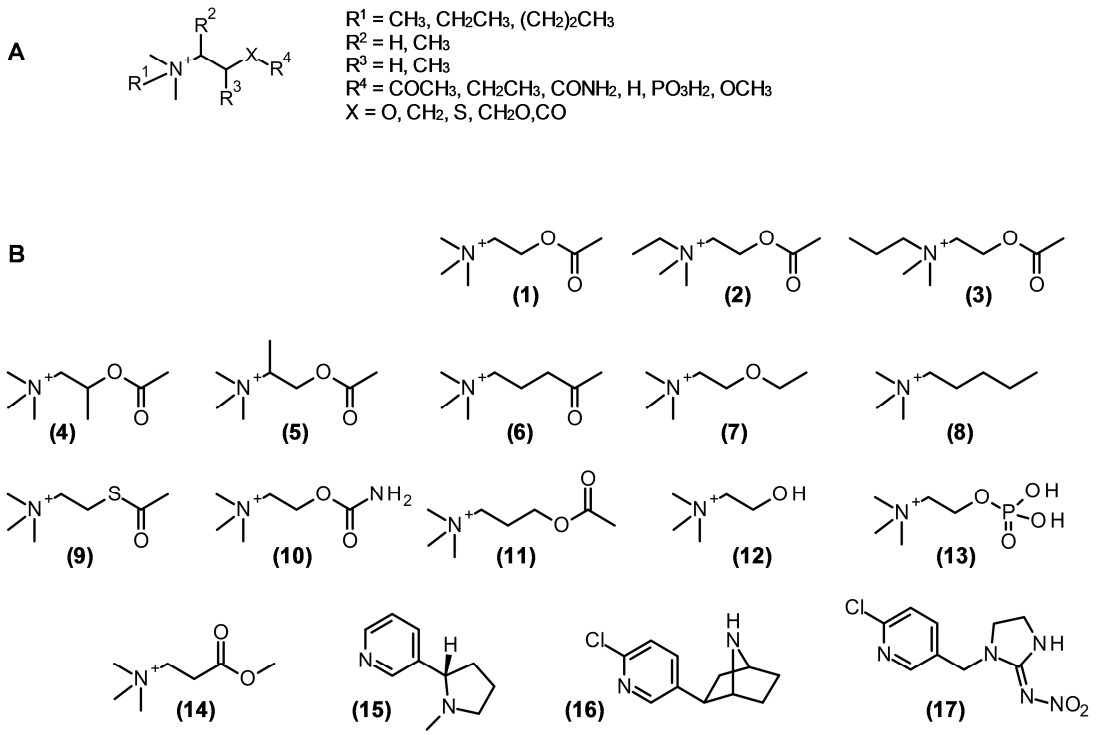


Figure 6: Chemical structures of acetylcholine (ACh) backbone derivatives for nAChR structure-activity relationships and of nicotinoids and imidacloprid. (A) Overview of ACh modifications. (B) Chemical structures: (1) ACh; (2) acetyl-N-ethylcholine; (3) acetyl-N-propylcholine; (4) acetyl-β-methylcholine; (5) acetyl-α-methylcholine; (6) trimethyl-(4-oxopentyl) ammonium; (7) trimethyl-ethoxyethyl ammonium; (8) trimethyl-pentyl ammonium; (9) acetyl-thiocholine; (10) carbamoylcholine; (11) acetyl-homocholine; (12) choline; (13) phosphocholine; (14) trimethyl-(3-methoxy-3-oxopropyl) ammonium; (15) (-)-nicotine; (16) (1R,2R,4S)-(+)-6-(6-chloro-3-pyridyl)-7-azabicyclo[2.2.1]heptane, epibatidine; (17) imidacloprid. From Dederer et al. 2013.

methyl ester (**14**). Reduction of the ester carbonyl oxygen in ACh (X) led to the choline ethyl ether (**7**), while simultaneous replacement of the ether oxygen by methylene resulted in trimethyl-pentyl ammonium (**8**). Replacing the acetyl methyl group in ACh (R^4) by amino led to carbamoylcholine (**10**). The nAChR agonistic potential of this collection was analysed on the insect α subunit-containing Lcα1/Ggβ2 and Lcα2/Ggβ2, and in comparison, on the chicken neuronal receptor Ggα4/Ggβ2.

The most remarkable differences with respect to the EC_{50} values were seen for acetyl-N-ethylcholine (**2**), which was as potent or more potent on Lcα1/Ggβ2 and Lcα2/Ggβ2 as ACh, while the EC_{50} value of (**2**) on Ggα4/Ggβ2 rose almost by a factor of 1000 compared to ACh. This might point to different steric conditions at the binding site of the quaternary ammonium group in insect and mammalian nAChRs.

Similarly, the EC_{50}s of acetyl-α-methylcholine (**5**) for Lcα1/Ggβ2 and Lcα2/Ggβ2 rose only moderately (factor 2.4) relative to ACh, while in the case of Ggα4/Ggβ2 the increase of EC_{50} for (**5**) was about 427-fold compared to ACh. In his review, Schmitt (2000) assumed that the α-methyl residue may interact at the same locus as the N-methyl groups. Based on this explanation, our results of (**5**) might again indicate the steric differences of quaternary ammonium group binding sites in insect and mammalian nicotinic receptors. By contrast, methyl substitution in β-position affected the EC_{50} values on all three receptors dramatically. In the case of acetyl-β-methylcholine (**4**), EC_{50} relative to ACh for Lcα1/Ggβ2 increased 287-fold, for Lcα2/Ggβ2 85-fold and for Ggα4/Ggβ2, the factor of increase was 830. The binding site conditions of insect, as well as vertebrate nAChRs apparently do not tolerate steric hindrance in β-position.

A third derivative where the difference in EC_{50} increases between insect and chicken receptors reached or exceeded the factor 100, was trimethyl-(3-methoxy-3-oxopropyl) ammonium (**14**), which basically corresponds to ACh with inverted ester function. Here, the EC_{50}s relative to ACh for Lcα1/Ggβ2 and Lcα2/Ggβ2 increased by modest 4.3 and 6.7-fold, while in the case of Ggα4/Ggβ2, this factor was 405. One explanation for these differences could be that the choline oxygen might perhaps play a bigger role in agonist binding of vertebrate than in insect nAChRs. Also it is possible that the carbonyl oxygen in inverted position encounters more steric hindrance in the binding pocket of vertebrate than of insect nAChRs.

Amongst the derivatives with less pronounced receptor potency differences, for acetylhomocholine (**11**) a dramatic increase of EC_{50} was noted for Ggα4/Ggβ2 (~550-fold) relative to ACh, while for the insect receptors Lcα1/Ggβ2 and Lcα2/Ggβ2, this was a more moderate 24-fold and 31-fold, respectively. Also trimethyl-(4-oxopentyl) ammonium (**6**) retained much of the ACh potency in Lcα1/Ggβ2 and Lcα2/Ggβ2 with EC_{50} increases of 6.3 and 2.4, respectively, relative to ACh, while the chicken receptor potency dropped by a factor of 89. Presumably, the binding site part of the two insect nAChRs addressing the area between the quaternary ammonium group and the ester function might be more tolerant to the chain length between these groups than the vertebrate nAChRs. Also, like in the case of (**14**), the choline oxygen is likely to be more important in interaction with the binging site of

mammalian than insect nAChRs. For all other derivatives differences of EC_{50} losses between the three receptor forms relative to ACh were generally near or below factor 10, which made these derivatives less instructive.

Structure-activity and structure-selectivity studies for agonist action on native and recombinant insect nAChRs have focussed in the past on neonicotinoid derivatives (Ihara et al. 2003, Millar and Denholm 2007, Shimomura et al. 2004, Thany et al. 2007, Tomizawa and Casida 2003, 2005, 2009) and on spinosyns (Baxter et al. 2010, Kirst 2010, Perry et al. 2007, Sparks et al. 2001). By contrast, to our knowledge, systematic structure-activity relationships for the natural ligand ACh as lead structure have not been reported for insect nAChRs.

In one study not published in a peer-reviewed journal, Schmitz (2002) systematically rigidized the lead structure of ACh upon 6 different structure types according to ligand classification of Schmitt (2000), and electrophysiologicaly tested these derivatives in structure-activity studies in *Xenopus* oocytes on four rat receptors: neuronal $\alpha3/\beta4$, $\alpha4/\beta2$ and $\alpha7$, as well as muscular nAChR. The results of the rat $\alpha4/\beta2$ receptor could be comparable with those of the chicken Ggα4/Ggβ2 since other studies could not show significant differences in sensitivity to nicotinoids and neonicotinoids between these two receptors (D'Amour and Casida 1999, Tomizawa et al. 2001, 2005). Schmitz (2002) investigated the effect of extension of the alkyl chain at the ACh quaternary ammonium and found out that the replacement of one methyl group by ethyl – independent of other structural changes of ACh – led to the loss of activity on rat $\alpha4/\beta2$ receptor and significant decrease of activity on other rat neuronal receptors. Additionally, from the ACh derivatives reported by Schmitz (2002), acetyl-α-methylcholine and acetyl-β-methylcholine were electrophysiologically tested in oocytes on $\alpha4/\beta2$. Schmitz (2002) observed that substitution in β-position affected the agonist potencies of derivatives: neither acetyl-β-methylcholine nor other β-substituted derivatives – independent from the length of substitution – were active on rat neuronal receptors. These results on rat $\alpha4/\beta2$ receptor might confirm our finding about steric conditions at the binding site in vertebrate nAChRs. In addition to *rac*-acetyl-α-methylcholine, Schmitz (2002) also tested *(R)*-acetyl-α-methylcholine and *(S)*-acetyl-α-methylcholine and found that the *(S)*-enantiomer has two times lower activity on rat $\alpha4/\beta2$ than the racemate, whereas the antipode was inactive on

that receptor. The higher activity of the racemate was explaned with the synergetic action of the two enantiomers (Schmitz 2002). Furthermore, acetyl-α-dimethylcholine was also found to be inactive on rat α4/β2 receptor (Schmitz 2002). Schmitt (2000) implied that the α-methyl residue of ACh derivatives may interact at the same locus in the binding site as the N-methyl groups. Additionally, the substitution on α position is demonstrated to be stereosensitive (Schmitt 2000).

In general, it appears that Ggα4/Ggβ2 is much less forgiving to the moderate structural changes on ACh that are displayed by the derivatives investigated in this study. EC_{50} increases were generally 2-3 orders of magnitude, except for the very close derivatives acetylthiocholine and carbamoylcholine. By contrast, the less dramatic or non-existent EC_{50} rises for the insect receptors Lcα1/Ggβ2 and Lcα2/Ggβ2 suggest that particularly substitution at the ACh quarternary ammonium, branching and the length of the ACh alkyl chain between ammonium and ester function, ketone analogs of ACh as well as the inverted ACh ester structure could be sources for selectivity between insect and chicken nAChRs investigated in this study and therefore logical entry points for derivatization programs.

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PAPER I

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Differential sensitivity of *Ctenocephalides felis* and *Drosophila melanogaster* nicotinic acetylcholine receptor α1 and α2 subunits in recombinant hybrid receptors to nicotinoids and neonicotinoid insecticides

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Abstract:

Nicotinic acetylcholine receptors (nAChRs) are the binding sites for nicotinoid drugs, such as nicotine and epibatidine, and are the molecular targets of the selectively insecticidal neonicotinoids. In this study we report the full length cDNA cloning of the three *Ctenocephalides* (*C.*) *felis* (cat flea) nAChR α subunits Cfα1, Cfα2, and Cfα3. When expressed in *Xenopus* oocytes as hybrid receptors with the *Gallus gallus* (chicken) β2 (Ggβ2) subunit, these cat flea α subunits formed acetylcholine-responsive ion channels. Acetylcholine-evoked currents of Cfα2/Ggβ2 were resistant to α-bungarotoxin, while those of Cfα1/Ggβ2 were sensitive to this snake toxin. The pharmacological profiles of Cfα1/Ggβ2, Cfα2/Ggβ2 and the chicken neuronal receptor Ggα4/Ggβ2 for acetylcholine, two nicotinoids and 6 insecticidal neonicotinoids were determined and compared. Particularly remarkable was the finding that Cfα1/Ggβ2 was far more sensitive to acetylcholine, nicotine and neonicotinoid agonists than either Cfα2/Ggβ2 or Ggα4/Ggβ2: for the anti flea neonicotinoid market compound imidacloprid the respective EC_{50}s were 0.02 μM, 1.31 μM and 10 μM. These results were confirmed for another insect species, *Drosophila melanogaster*, where the pharmacological profile of the Dmα1 and Dmα2 subunits as hybrid receptors with Ggβ2 in *Xenopus* oocyte expressions resulted in a similar sensitivity pattern as those identified for the *C. felis* orthologs. Our results show that at least in a Ggβ2 hybrid receptor setting, insect α1 subunits confer higher sensitivity to neonicotinoids than α2 subunits, which may contribute *in vivo* to the insect-selective action of this pesticide class.

1. Introduction

Nicotinic acetylcholine receptors (nAChRs) are ligand-controlled cation channels, that act in fast neurotransmission at cholinergic synapses in vertebrates (Dolly and Barnard 1984, Galzi et al. 1991, Corringer et al. 2000, Kalamida et al. 2007) and invertebrates (Jones and Sattelle 2004, Jones et al. 2007, Thany et al. 2007). They belong to the cysteine (Cys) loop ligand-gated ion channel (LGIC) superfamily and form pentamers of subunits, each of which consists of a N-terminal Cys loop-containing ligand-binding extracellular domain, four transmembrane helices and a C-terminus facing the extracellular space (Sine and Engel 2006). Two vicinal cysteine residues in the extracellular domain, which are involved in acetylcholine (ACh) binding, define nAChR α subunits in all species, while non-α subunits (β, γ, δ or ε) lack this motif (Ortells and Lunt 1995, Le Novere and Changeux 1995, Karlin 2002, Connolly and Wafford 2004, Barry and Lynch 2005).

In mammals, seventeen nicotinic acetylcholine receptor subunits (α1-α10, β1-β4, γ, δ, ε) coassemble into mostly heteropentameric, sometimes homopentameric, nAChRs that are present at the skeletal neuromuscular junction, the autonomic ganglia and the central nervous system (Millar and Gotti 2009, Albuquerque et al. 2009). In humans, nAChRs represent valuable drug targets to treat disorders ranging from neuropathic pain, Alzheimer's disease and schizophrenia to depression and tobacco addiction (Jensen et al. 2005, Livett et al. 2006, Taly et al. 2009, D'hoedt and Bertrand 2009).

Insects possess fewer nAChR subunit genes than mammals, with e. g. 10 genes in *Drosophila* (*D.*) *melanogaster* (Dmα1-Dmα7, Dmβ1-Dmβ3, Littleton and Ganetzky 2000, Sattelle et al. 2005) and *Anopheles* (*A.*) gambiae (Agα1-Agα9, Agβ1, Jones et al. 2005), 11 genes in *Apis* (*A.*) *mellifera* (Amα1-Amα9, Amβ1-Amβ2, Jones et al. 2006), and 12 genes in *Bombyx mori* (Bmα1-Bmα9, Bmβ1-Bmβ3, Shao et. al. 2007). In further contrast to mammals, the expression of functional nAChRs in insects appears to be restricted to the central nervous system (Sattelle 1980, Breer and Sattelle 1987). Nicotinic acetylcholine receptors are targets for different classes of insecticides, such as nicotine, spinosyns or nereistoxin analogs (Millar and Denholm 2007). The by far largest and commercially most important insecticide class addressing nAChRs are the neonicotinoids (Fig. 1), that have entered the market as

pesticides in 1991 and have experienced a fast growth in sales (Millar and Denholm 2007, Jeschke and Nauen 2008). It has been shown by electrophysiological experiments on nerve preparations from *D. melanogaster*, *Periplanata americana*, *Apis mellifera* and *Heliothis virescens* (Brown et al. 2006, Tan et al. 2007, Thany et al. 2007), that neonicotinoids such as imidacloprid or clothianidin (Fig. 1) act as partial or full agonists in an *in vivo* setting. The low toxicity of neonicotinoids to mammals has been explained by their rather poor agonist action on most mammalian nAChRs, as opposed to their strong agonist potency on insect nAChRs (Tomizawa and Casida 2003, 2005, 2009; Matsuda et al. 2001, 2005, 2009).

The molecular definition of the neonicotinoid target(s) in insects has been hampered by the difficulty of heterologous functional expression of insect nAChRs. Successful examples are largely limited to the formation of trans-species hybrid heteropentamers of insect α subunits with either chicken or rat β2 subunits (Millar 1999, 2009). *Xenopus* oocyte expression and electrophysiology experiments with hybrid nAChR consisting of *D. melanogaster* Dmα2 and chicken β2 (Ggβ2) subunits suggested partial agonist activity of imidacloprid and super-agonist activity of clothianidin (Matsuda et al. 1998, Ihara et al. 2004). A comparative *Xenopus* oocyte electrophysiology study confirmed for Dmα2/Ggβ2 hybrids the partial agonist activity of imidacloprid and demonstrated the full agonist activity of nitenpyram. By contrast, only nitenpyram proved to be a partial agonist for Dmα1/Ggβ2, while imidacloprid showed no agonist activity in this receptor combination (Ihara et al. 2003). Radioligand binding experiments on diverse *Myzus persicae* α (Mpα) subunits coexpressed with rat β2 subunit in *D. melanogaster* S2 cells showed high affinity of [^{3}H]imidacloprid for the rat β2 combinations with Mpα2 and Mpα3, but not Mpα1 and Mpα4 (Huang et al. 1999). Similar radioligand binding studies in S2 cell as well as *Xenopus* oocyte electrophysiology studies of rat β2 subunit coexpression experiments with *Nilaparvata (N.) lugens* α (Nlα) subunits showed that Nlα1/rat β2 forms a high affinity receptor, Nlα2/rat β2 a low affinity receptor, while Nlα3/rat β2 or Nlα4/rat β2 constitute no neonicotinoid receptors (Liu et al. 2005, 2006, 2009). *Xenopus* oocyte coexpression of Nlα8 with rat β2 yielded also a low affinity neonicotinoid-gated ion channel, whose affinity could be increased 40-fold by coassembly with Nlα3 (Yixi et al. 2009). Sequence analyses on neonicotinoid-

resistant insect mutants and heterologous expression of the mutated gene products have implicated amino acid exchanges in the insect α1 subunit (*N. lugens*, Liu et al. 2005; *D. melanogaster*, Perry et al. 2008), the α3 subunit (*N. lugens*, Liu et al. 2005) and the β2 subunit (*D. melanogaster*, Perry et al. 2008) as potential causes for neonicotinoid resistance. However, taken together, the available data on insect nicotinic receptor neonicotinoid interactions within and between different species cannot easily be aligned and remains contradictory.

The insect veterinary parasite *Ctenocephalides* (*C.*) *felis* (cat flea) is a major source of discomfort to cats and dogs and their owners, which leads to expenditures well over 2 billion US$ annually for control measures. Neonicotinoids, specifically imidacloprid, nitenpyram and dinotefuran (Fig. 1), are main weapons to combat cat fleas (Rust and Dryden 1997, Rust 2005). Given the economic importance of cat fleas, it is surprising that only a single report on the potential molecular target(s) of neonicotinoids in *C. felis* has been published. Bass et al. (2006) have reported the partial gene cloning, largely of the extracellular domains, of 8 nicotinic receptor subunits from *C. felis* (Cfα1-Cfα4, Cfα7, Cfα8, Cfβ1). Chimeric receptors of the ligand binding domains of Cfα1, Cfα2, Cfα3, Cfα7 and Cfβ1 with the *D. melanogaster* Dmα2 endoplasmic reticulum import signal sequence, transmembrane helices and C-terminus were coexpressed with rat β2 subunitin S2 cells and investigated by [^{3}H]imidacloprid ligand binding studies. The two functional combinations containing Cfα1 and Cfα3 sequences did bind the radioligand with high affinity (Bass et al. 2006).

In this study, we have identified the full length cDNA sequences of the Cfα1, Cfα2 and Cfα3 genes and we have cloned the genes for their *D. melanogaster* orthologs Dmα1, Dmα2 and Dmα3, as well as chicken α4 (Ggα4) and β2 (Ggβ2). We have demonstrated the functional coexpression of all these subunits with chicken β2 in *Xenopus* oocytes in two electrode voltage clamp experiments. In *Xenopus* oocytes coexpressing Cfα1/Ggβ2, Cfα2/Ggβ2, Dmα1/Ggβ2, Dmα2/Ggβ2, and, for comparison, the chicken CNS nicotinic acetylcholine receptor Ggα4/Ggβ2, agonist properties, effective concentrations 50% (EC_{50}) values and the electric current signal strength relative to that elicited by ACh (I_{max}[%ACh]) were determined for the natural ligand ACh, for the nicotinoids nicotine and epibatidine, as well as for the

neonicotinoids imidacloprid, nitenpyram, acetamiprid, thiacloprid, dinotefuran and clothianidin (compare Fig. 1). Furthermore, the antagonist properties of α-bungarotoxin on these nAChR combinations were investigated. Our results suggest that the α1 subunits of both insects in hybrid receptors with chicken β2 confer far higher sensitivity to neonicotinoid agonist action than their insect α2 counterparts.

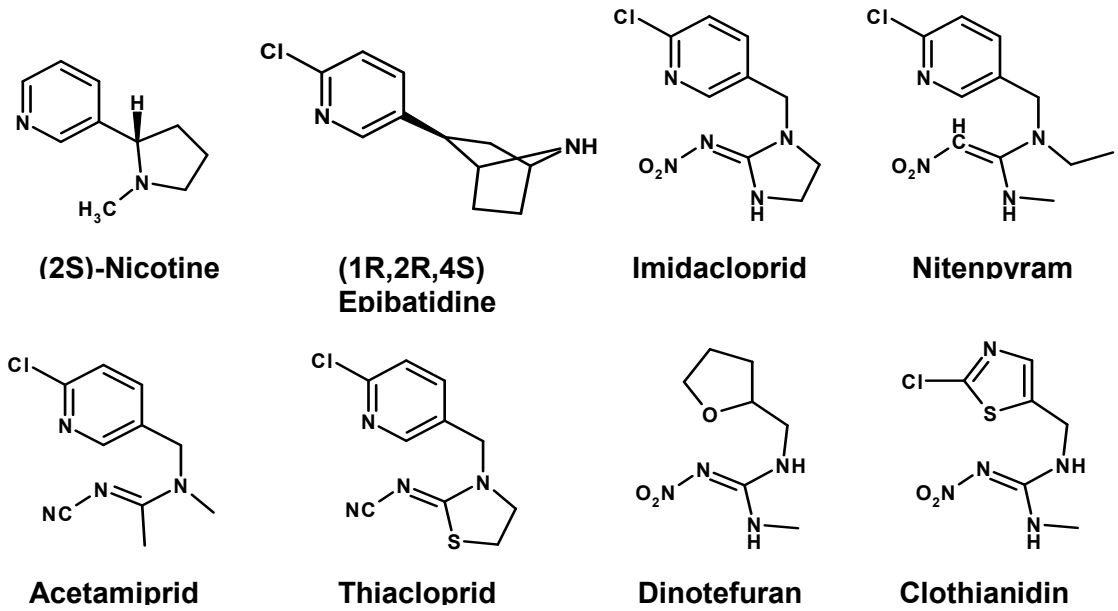

Figure 1: Chemical structures of nicotinoids and neonicotinoids applied in this study.

2. Materials and Methods

2.1 Bacterial strains, plasmids, chemicals and insects.

Bacterial cultures were grown in Luria-Bertani (LB) medium modified with supplements as required by the bacterial background and the introduced resistance genes. Polymerase chain reaction (PCR) products were cloned into pCR2.1-Topo, introduced into *Escherichia* (*E.*) *coli* Top10 cells (Invitrogen) and subcloned into the mammalian expression vector pcDNA3.1(+) (Invitrogen). Acetylcholine (ACh), nicotine and epibatidine were from Sigma. Imidacloprid (Dr. Ehrenstorfer), nitenpyram, thiacloprid (Riedel-de Haen) acetamiprid (Annopol), and dinotefuran (Nissan) were from external sources, clothianidin was provided by Intervet

Singapore. *C. felis* adult fleas (adapted to artificial feeding, Wade & Georgi, 1988) used in this study were collected from long term cultures maintained at Intervet Innovation GmbH, Schwabenheim, Germany.

2.2 Identification, isolation and phylogenetic analysis of nicotinic acetylcholine receptor subunit genes from chicken, *D. melanogaster* and *C. felis*

Total RNA was extracted from *D. melanogaster* imagoes, from *C. felis* flea imagoes and from freshly prepared chicken brain by a modification of the guanidinium thiocyanate/phenol extraction method (Chomczynski and Sacchi, 1987; Trizol, Sigma). Other molecular biology techniques were performed essentially as described by Sambrook and Russell (2001). Reverse transcription (RT-) PCR was performed using the Titan one tube RT-PCR system (Roche) with total RNA (0.5 – 1 μg/50 μl) as template. In some cases, reverse transcription of total RNA was performed in a separate step followed by PCR under various conditions.

The chicken nicotinic acetylcholine receptor subunit genes α4 and β2 (*Ggα4*, *Ggβ2*) were isolated by RT-PCR with chicken brain total RNA as template and the primer pairs GGGTACCATGGGATTTCTCGTGTCGAAGGGAACC/GGCGGCCGCTTAGATCATTCCTGCCAGCCACGG and GGAATTCGGTACCATGGCGCTGCTCCGCGTCCTCTGCC/GAAGCTTATTTGGAGGTGGGGGTGCCCTG, respectively.

In the case of the *D. melanogaster* nicotinic acetylcholine receptor subunit genes α1, α2 and α2 (*dmα1*, *dmα2, dmα3*), the primer pairs CCCGGGTACCATGGGTAGCGTGCTATTCGCAG/CTTAAGCTTCTATAAGGTGTTCTCGCTGCC,
CCCGGGTACCATGGCTCCTGGCTGCTGCACC/CTTAAGCTTTAATTCTTCTTCTCGGTTAGATTG and
CTGGATCCGGTACCATGAAGTGGTTTCAAGTGACCATAG/CTAAGCTTACAACACGGGCCTGGCCAC, respectively, were used in RT-PCRs with fly imago total RNA as template. The PCR products obtained in these reactions were cloned into pCR2.1-Topo and sequenced.

For the cloning of the genes encoding the nicotinic acetylcholine receptor subunits α1, α2 and α3 from *C. felis* (*cfα1*, *cfα2* and *cfα3*), rapid amplification of cDNA ends was performed based on our own degenerate primer RT-PCR data (not shown) and on the published partial cDNA sequences DQ237865, DQ237866 and DQ237867 (Bass et al. 2006). The missing cDNA sequences of *cfα1*, *cfα2* and *cfα3* were obtained by 3'-RACE (specific primers CGGGATCGATCTGCAGGATTACTACATCAG, GATATCTTGGGGGTCCCAGCCGAAAGGCATG, TGGGATATACTAGAAGTTCCGGCTGTCAGG, for *cfα1*, *cfα2* and *cfα3*, respectively) and 5'-RACE (specific primers: CGGTGTGATGCAGAATCGCCTTCGTCATG, GATATCCCACTCTACACTGGGATAATACTCCCTC, CTTGACTGTGAGAGCGTCAGTAACATTCACC, for *cfα1*, *cfα2* and *cfα3*, respectively) using total RNA from flea imagoes as templates and the SMART RACE cDNA Amplification Kit (Clontech) with generic flanking 5'- and 3'-RACE primer (Clontech), as outlined by the manufacturer. Based on the deduced start and stop codon positions in the 5'- and 3'-RACE product sequences, the PCR primers GCGGATCCAAAATGGAGAGCCTGTTACTGGCGCTC and GCAAGCTTTTATAGTTCCCCTGTGCCCATTTTAAGCAG for *cfα1*, CAGGTACCATGTATCTTACTAAATCTGCTCGG and CTCTCGAGTTATACATCTGGTAAAAACTGTTGTTGG for *cfα2*, as well as GAGGTACCATGAGGCTCCGTCCACCGGACG and GTCTCGAGTTATAGCTTGACATGTACATTAGC for *cfα3*, were then designed for the RT-PCR amplification of the full length genes from adult flea total RNA. The restriction enzyme sites introduced by the primers are underlined. The PCR products were cloned into pCR2.1-Topo and sequenced. Thereby, from the first RT-PCR amplification a 1747 bp PCR product with a 1728 bp open reading frame (*cfα1*) encoding *C. felis* nicotinic acetylcholine receptor subunit α1 (Cfα1) was identified, while the second RT-PCR yielded a 1702 bp PCR product with a 1686 bp open reading frame (*cfα2*) encoding *C. felis* nicotinic acetylcholine receptor subunit α2 (Cfα2) and the third RT-PCR gave a 1918 bp PCR product with a 1902 bp open reading frame (*cfα3*) encoding *C. felis* nicotinic acetylcholine receptor subunit α3 (Cfα3). Consensus sequences devoid of PCR errors were identified by sequencing of 3 independent plasmid-cloned PCR fragments and direct PCR product sequencing

(*cfα1* and *cfα2*), or 5 independent plasmid-cloned PCR fragments (*cfα3*), and by performing ClustalW alignments with the translated DNA sequences. ClustalW multiple sequence alignments of *C. felis* nicotinic acetylcholine receptor α subunits and other insect receptor subunits, the generation of phylogenetic trees and bootstrap analyses were done with in the DNAStar Lasergene software package. Bioinformatics analysis for the presence of endoplasmic reticulum import sequences and transmembrane helices were performed using SignalP3.0 (http://www.cbs.dtu.dk/services/SignalP/) and TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/), respectively.

2.3. Xenopus laevis oocyte expression and electrophysiology experiments with nicotinic acetylcholine receptor subunit genes from chicken, *D. melanogaster* and *C. felis*

The open reading frames of Ggα4, Ggβ2, Cfα1-3 and Dmα1-3 were subcloned into pcDNA3.1(+) downstream of the T7 promoter *via* restriction enzyme sites introduced by the PCR primers. The resulting plasmids were linearized by Not I (Dmα1, Cfα1), Hind III (Cfα2, Cfα3), EcoR I (Ggβ2), Spe I (Dmα3) or Xba I (Dmα2, Ggα4), and *in vitro* transcription to obtain 5'-capped cRNA, and subsequent polyadenylation was performed using the mMESSAGE mMACHINE T7 transcription kit (Ambion) according to the manufacturers protocol. Transcripts were recovered by LiCl precipitation, dissolved in nuclease-free water at a final concentration of ~ 2 μg/μl, and stored at -80^{0}C until use.

Defolliculated *Xenopus laevis* oocytes (sates V-VI) were purchased from Ecocyte Biosciences. 50.6 nl cRNAs were injected in a 1:1 molar ratio using a micromanipulator (World precision instruments). The oocytes were incubated for 48-96 h at 17^{0}C in modified Barths solution (5 mM Hepes pH 7.2, 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 2.4 mM Na-pyruvate, PS). Oocytes held in bath were perfused with Barths solution (5 mM Hepes pH 7.2, 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$) at a flow rate of approximately 1.8 ml/min were voltage clamped at – 60 mV using the two electrode clamp mode of an Turbo Tec-03x amplifier (NPI electronic). Electrodes were pulled from borosilicate glass (Science

products) using the Puller PC-10 (Narishige group), and filled with 3 M KCl. The electrode resistance ranged between 1-5 MΩ on the current-passing side. Agonist solutions, freshly prepared in Barths solution from dimethylsulphoxide- (DMSO-) dissolved stocks (10 mM – 500 mM), but not exceeding 1,5% (v/v) DMSO, were applied via bath perfusion for 30 sec. The resulting inward current was recorded using CellWorksLite 5.5.1 (NPI electronic), and analyzed later. An interval of 2 min was routinely maintained between agonist applications, which was elongated in some case to up to 10 min. Dose-response curves were analyzed using the Hill equation ($I = I_{max} ([A]^{nH}/[A]^{nH} + EC_{50}{}^{nH})$. Hereby "I" represents the current response measured at its peak, "I_{max}" the maximum response in the experiment, "EC_{50}" the agonist concentration for 50% maximum response, and "n_H" the Hill coefficient. and Calculations of these parameters as well as standard deviations (SD) and *p* values (Student's *t*-test) were done using XL-fit (Microsoft®Excel™). For I_{max}[% ACh] determinations of nicotinoid and neonicotinoid agonists on different nAChR subunit combinations, a saturating ACh concentration was applied first, the maximum current recorded, and then saturating test agonist concentrations were applied. The percentage of maximum current (I_{max}[% ACh]) relative to ACh was calculated.

3. Results

3.1 Isolation of chicken, *D. melanogaster* and *C. felis* genes encoding for nicotinic acetylcholine receptor subunits, and bioinformatic analysis.

The chicken nicotinic acetylcholine receptor genes α4 and β2 (*Ggα4*, *Ggβ2*) were isolated from chicken brain total RNA by RT-PCR. Their deduced translation products were identical to the Genbank database sequences AJ250361 and AJ250362, respectively. For the PCR amplification of the *D. melanogaster* nicotinic acetylcholine receptor subunits α1, α2 and α3 (*dmα1*, *dmα2*, *dmα3*) total *D. melanogaster* RNA was used. The deduced translation product of *dmα1* was identical to the Genbank sequence NM_079757 (Bossy et al. 1988, ALS) and the encoded polypeptide sequence of *dmα2* proved to be identical to that of X53583 (Sawruk et al. 1990, SAD). By contrast, the sequence of our *dmα3* gene isolate

(submitted to Genbank with the accession number FR689750) contained, compared to Genbank-deposited *dmα3* sequences (Y15593 and NM_080340; Schulz et al. 1998), a deletion present in several independently isolated cDNA clones, that led at the protein level to the loss of a poly-alanine region predicted to be located in the second cytoplasmic loop.

In case of *C. felis*, Genbank-deposited partial DNA sequences encoding the ligand-binding domains of the nicotinic acetylcholine receptor subunits α1, α2 and α3 (*cfα1*, *cfα2*, *cfα3*) but lacking the sequences of the 5'-ends, the transmembrane domains and the 3'-ends (Bass et al. 2006) were used to design primers for 5'- and 3'-RACE PCR experiments. Thereby, the missing 5'- (FR689741, FR689744, FR689747, respectively) and 3'-cDNA sequences (FR689742, FR689745, FR689748, respectively) of the *C. felis* α1-, α2- and α3-encoding genes were identified. Based on the deduced start and stop codon information, the full length genes (*cfα1*, *cfα2*, *cfα3*) of the three subunits were isolated by RT-PCR from *C. felis* total RNA. The first product *cfα1* contained a 1728 bp open reading frame (FR689743). The deduced polypeptide sequence of this *C. felis* gene, Cfα1, showed extensive sequence identity to nicotinic receptor α-subunits from other insect species, particularly to *A. gambiae* α1 (80.4%, AY705394), *D. melanogaster* α1 (79.8%, NM_079757), and *A. mellifera* α1 (77.0%, DQ026031). The RT-PCR product of *cfα2* contained a 1686 bp open reading frame (FR689746). The deduced polypeptide sequence of this second *C. felis* α gene was highly homologous to α2 subunits from *A. gambiae* α2 (84.9% identity, AY705395), *A. mellifera* α2 (83.9% identity, NM_001011625) and *Tribolium* (*T.*) *castaneum* α2 (83.1% identity, EF526081). The third full length *C. felis* α gene *cfα3* encompassed a 1902 bp open reading frame (FR689749), and showed high sequence identity to α3 subunits from *A. mellifera* α3 (80.3%, DQ026032), *T. castaneum* α3 (78.7%, EF526082) and *D. melanogaster* α3 (73.3%, Y15593). More detailed polypeptide sequence comparisons, as illustrated in a multiple sequence alignment (Fig. 2) and the DNAStar/ClustalW-based molecular tree (Fig. 3) confirmed that *cfα1*, *cfα2*, *cfα3* belong to the insect nicotinic receptor subunit α1, α2 and α3 families, respectively.

Inspection of the consensus sequence of the Cfα1-3 multiple sequence alignment (Fig. 2) revealed the presence of the cysteine loop and the loop A-C motifs typical for nicotinic receptor α subunit ligand binding sites within the putative extracellular domains in all three polypeptides (Arias 1997, Corringer et al. 2000, Albuquerque et al. 2009). These loops contained a number of highly conserved amino acid residues: within the cysteine loop the two cysteines corresponding to C^{128} and C^{142} in the reference Torpedo α subunit, in loop A the amino acid residues corresponding to tryptophane W^{86} and tyrosine Y^{93}, in loop B residues corresponding to W^{149} and Y^{151} and within loop C amino acids corresponding to C^{192} and C^{193} as well as Y^{190} and Y^{198} (all Torpedo α subunit numbering; Arias 1997, Corringer et al. 2000, Albuquerque et al. 2009).

Kyte-Doolittle hydrophobicity plots of Cfα1-3 suggested the presence of an N-terminal endoplasmic reticulum (ER) import signal sequence and of several transmembrane regions in all three polypeptide sequences (Fig. 4). Analysis of the three *C. felis* nicotinic receptor α subunit sequences with Signal P3.0 showed for Cfα1 an ER import signal sequence probability of 1.000 with a predicted cleavage site between A^{21} and N^{22}, for Cfα2 a probability of 0.888 with a predicted cleavage site between C^{27} and N^{28}, and for Cfα3 a probability of 0.996 with a predicted cleavage site between G^{29} and N^{30} (see Fig. 2). Further analysis of the Cfα subunit sequences identified in this study with the transmembrane helix detection program TMHMM predicted in all three gene products the four transmembrane helices typical for nicotinic acetylcholine receptor subunits and ligand-gated ion channels (LGIC) in general (Fig. 4; Arias 1997, Corringer et al. 2000, Albuquerque et al. 2009)). In the case of Cfα2, an additional transmembrane helix (T^{428}-M^{450}) not typical for LGICs was predicted (Fig. 4). Within and neighbouring to the second transmembrane helices TM2, the highly conserved amino acid residues of the nicotinic acetylcholine receptor ion channel 'rings' corresponding to D^{234}, E^{237}, S^{240}, T^{244}, L^{247}, V^{251}, L^{254} and E^{258} (chicken α7 subunit numbering, Corringer et al. 2000) are either also conserved in Cfα1-3, or replaced by structurally and/or functionally similar amino acids (Fig. 2; S^{240}➔A, Cfα2; T^{244}➔S, Cfα1-3; V^{251}➔M, Cfα2, chicken α7 numbering, Corringer et al. 2000).

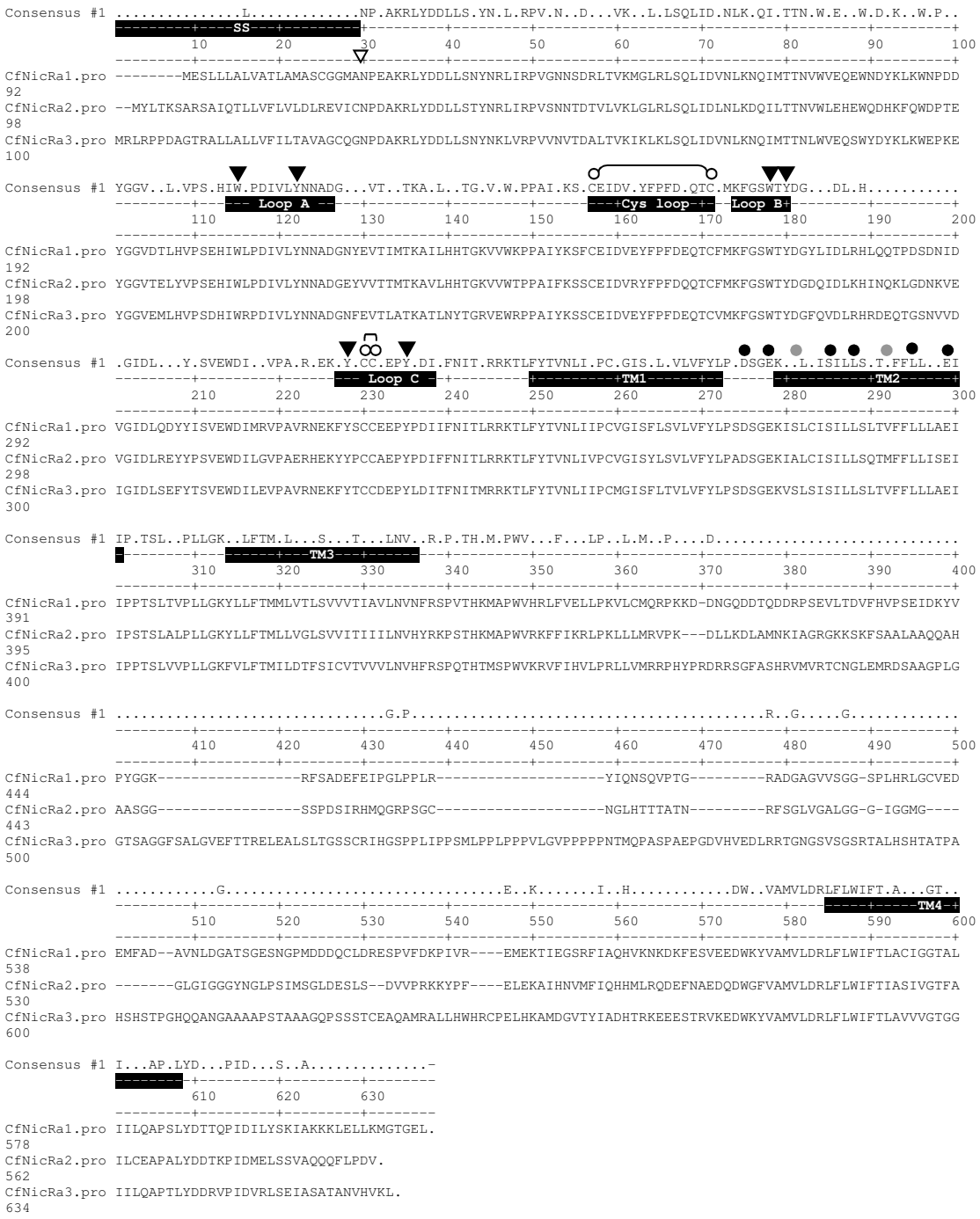

Figure 2: ClustalW amino acid sequence alignment of *C. felis* nAChR α subunits. Predicted ER import signal sequences (SS), the conserved extracellular loops (loop A, Cys loop, loop B, loop C) and the four predicted transmembrane helices (TM1-TM4) are highlighted by black bars under the consensus sequence. The predicted cleavage sites for the ER signal peptidase is indicated by an open triangle. The conserved tyrosine and tryptophane residues within the loop structures are marked with filled triangles, while the half-cystines of the Cys loops and the ligand binding sites are highlighted by connected open circles. The conserved amino acid residues forming the nAChR ion channel 'ring' in and near TM2 (Corringer et al. 2000) are highlighted by black circles (full conservation) or grey circles (partial conservation).

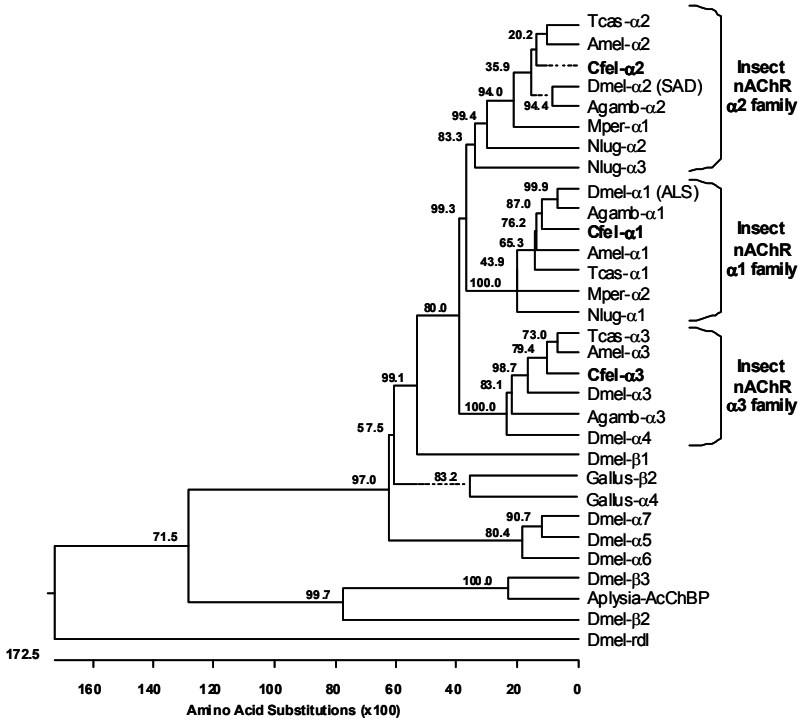

Figure 3: Phylogenetic dendrogram of insect nicotinic acetylcholine receptor subunits. The dendrogram (DNAStar) was derived from ClustalW-aligned protein sequences of Cfα1 (FR689743), Cfα2 (FR689746) and Cfα3 (FR689749) identified in this study together with the 10 *D. melanogaster* subunits (Dmel-α1-α7, Dmel-β1-β3; accession No: NM_079757, X53583, Y15593, AJ272159, AF272778, AJ554209, AJ554210, NM_079203, NM_170147, NM_164386, respectively) and the α1-3 sequences of *Anopheles gambiae* (Agamb; accession No: AY705394, AY705395, AY705396, respectively), *Apis mellifera* (Amel; accession No: DQ026031, NM_001011625, DQ026032, respectively), *Tribolium castaneum* (Trib; accession No: EF526080, EF526081, EF526082, respectively) *Nilaparvata lugens* (Nlug; accession No: AY378698, AY378702, AY378700, respectively) and the α1-2 sequences from *Myzus persicae* (Mper, accession No: X81887 and X81888, respectively). Furthermore, for reference, *Gallus gallus* (chicken) α4 and β2 (Gallus; accession No: AJ250361 and AJ250362, respectively) and *Aplysia californica* acetylcholine binding protein (Aplysia-AcChBP; accession No: AF322877) were included. *D. melanogaster* GABA-gated chloride channel (Dmel-rdl; accession No: M69057) served as an outgroup.

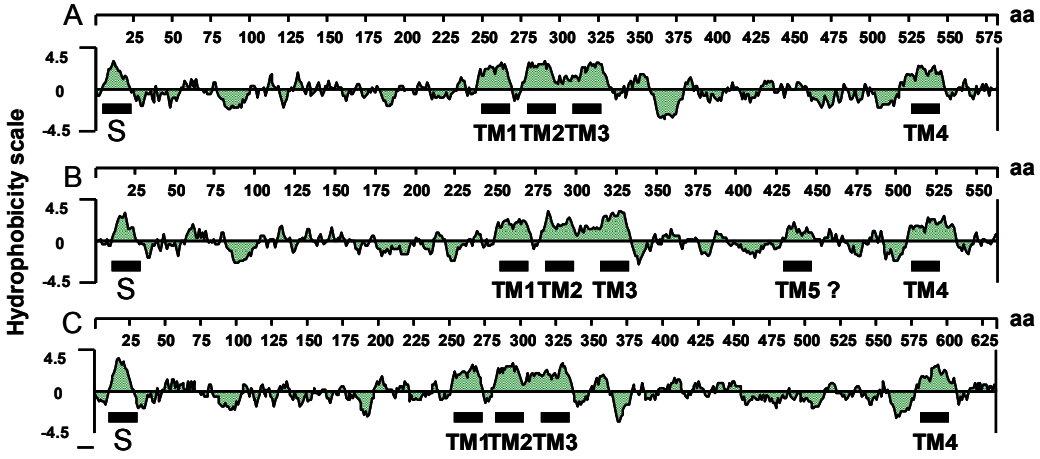

Figure 4: Kyte-Doolittle hydrophobicity plots of Cfα1 (A), Cfα2 (B) and Cfα3 (C). The predicted ER import signal sequences (S), four predicted transmembrane helices (TM1-TM4) of Cfα1-3 and the predicted fifth transmembrane helix of Cfα2 (TM5) are indicated by underlying bars.

3.2. Functional studies by *Xenopus laevis* oocyte expression and voltage clamp electrophysiology with the specific ligand acetylcholine

To examine the functional properties of the full length Cfα1-3 cDNAs identified in this study, they were coexpressed with the chicken β subunit Ggβ2 (Bertrand et al. 1994) and examined by two-electrode voltage clamp assays. In all three cases, application of ACh led to dose-dependent currents, which were rather weak in the case of Cfα3/Ggβ2, but strong in the case of Cfα1/Ggβ2 and Cfα2/Ggβ2 (Fig. 5A). This result confirmed that all three full-length *C. felis* cDNAs identified in this study encode functional α subunits. In the case of Cfα2/Ggβ2, the EC_{50} for ACh was 9.0 μM with a Hill coefficient of 2.2, suggesting positive cooperativity (Fig. 5D, table 1). Cfα1/Ggβ2 was 2 orders of magnitude more sensitive to ACh activation (EC_{50} = 50 nM, Fig. 5C, table 1), and a Hill coefficient of 1.03 suggested that this subunit combination does exhibit no cooperativity (table 1). For Cfα3/Ggβ2, an EC_{50} for ACh could not be determined in *Xenopus* two electrode voltage clamp experiments, due to the low signals, which also precluded detailed analysis of other agonists used in this study. A similar picture as for Cfα1-Cfα3 was seen in *Xenopus* two electrode voltage clamp experiments for the corresponding *D. melanogaster* orthologs: for Dmα2/Ggβ2, strong ACh-elicited currents and an EC_{50} of 6.6 μM were detected, with a Hill coefficient of 1.7 (table 2). The combination Dmα1/Ggβ2 also gave strong currents upon ACh exposure and proved to be much more sensitive than the Dmα2 combination, with an EC_{50} of 70 nM and a Hill coefficient of 1.01 (table 2). By contrast, Dmα3/Ggβ2 gave only small ACh-dependent currents, precluding not only EC_{50} determination for this ligand, but also for other agonists used in this study (not shown).

Cfα2/Ggβ2 was insensitive to α-bungarotoxin at 2 μM (Fig. 5B), while ACh-induced currents of the Cfα1/Ggβ2 combination were completely blocked by this concentration of snake toxin (Fig. 5B). Further experiments showed that the latter receptor is sensitive to much lower α-bungarotoxin concentrations, with 100 nM yielding > 60% blockage (data not shown).

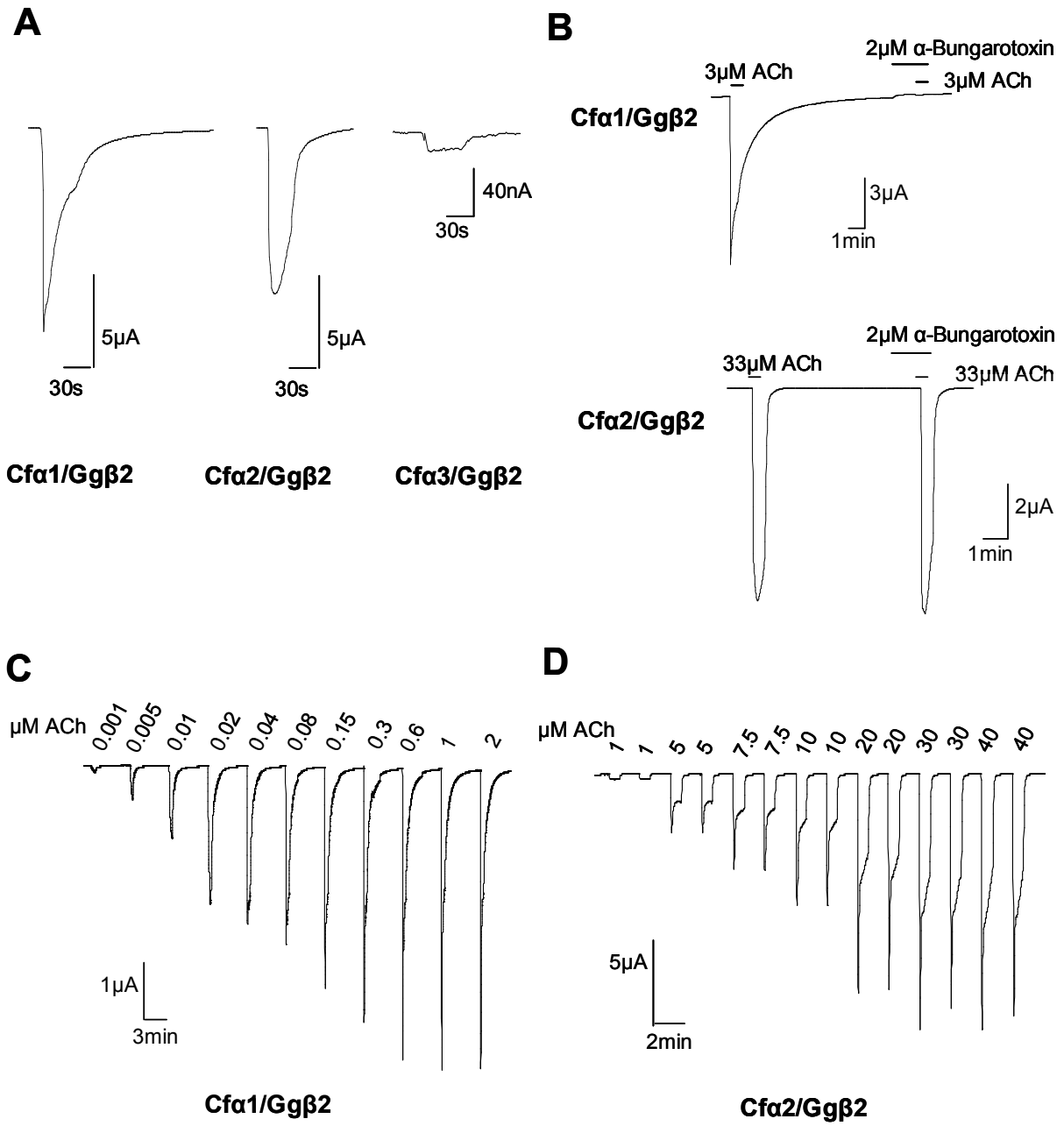

Figure 5: Current responses of nAChRs in *Xenopus* oocyte two electrode voltage clamp experiments – ACh. (A) Cfα1, Cfα2 and Cfα3. (B) α-bungarotoxin sensitivity of the ACh response of Cfα1 and Cfα2. (C) ACh titration of the current response of Cfα1. (D) ACh titration of the current response of Cfα2.

3.3. Comparative nicotinoid and neonicotinoid pharmacology of *C. felis* and *D. melanogaster* α1 and α2 hybrid receptors and *G. gallus* CNS α4/β2 receptor

Cfα2/Ggβ2 and Dmα2/Ggβ2: all nicotinoid and neonicotinoid ligands (Fig. 1) tested were agonists for this subunit combination in the μM concentration range. For all ligands clear dose-response relationships (see Fig. 6B, Fig. 7 A,B) could be documented and analyzed, with the exception of thiacloprid. The agonist potencies increased in the following order: dinotefuran < nitenpyram < nicotine ~ acetamiprid < clothianidin < imidacloprid < epibatidine (table 1). I_{max} determinations with ACh as standard showed that clothianidin behaved as a superagonist, dinotefuran and epibatidine as a full agonists, and all the other ligands were partial agonists, with acetamiprid being the least efficient (table 1). Like for the natural ligand ACh, the Hill coefficients for the two nicotinoids and 5 neonicotinoids were close to 2, which indicates positive cooperativity. Thiacloprid (Fig. 1) was also an agonist of Cfα2/Ggβ2, but EC_{50} or I_{max} values could not be determined, because in contrast to other neonicotinoids, repeated application of this derivative led to a continuous loss of signal, even at low concentrations (e. g. 1 μM, data not shown). The *D. melanogaster* ortholog Dmα2/Ggβ2 combination showed a similar picture as Cfα2/Ggβ2 for all ligands considered in both absolute (EC_{50}) and relative potency, except for clothianidin, which had a 3-fold higher EC_{50} in the *Drosophila* receptor (5.5 μM *versus* 1.65 μM, see table 2 in comparison with table 1, Fig. 7C,D).

Cfα1/Ggβ2 and Dmα1/Ggβ2: the Cfα1/Ggβ2 hybrid subunit combination was also responsive to all nicotinoid and neonicotinoid ligands (Fig. 1) tested, but the sensitivity of this receptor to these agonists was generally one or two orders of magnitude higher compared to Cfα2/Ggβ2. Even low epibatidine (Fig. 6C) and thiacloprid (not shown) concentrations led to a persistent activation of Cfα1/Ggβ2, which precluded the determination of proper dose response curves. The EC_{50} values for nicotinoid and neonicotinoid ligands were generally in the nanomolar range, except for dinotefuran, whose EC_{50} was 4.19 μM, and showed an increase in potency with the order dinotefuran < nitenpyram < clothianidin < acetamiprid < nicotine < imidacloprid (table 1). Particularly potent was the insecticide imidacloprid, with an EC_{50} of 20 nM (table 1). The Hill coefficients were between 0.96 and 1.29, suggesting that none of the ligand-receptor interactions analyzed here displays pronounced

cooperativity. All nicotinoid and neonicotinoid ligands investigated here were partial agonists on Cfα1/Ggβ2 compared to the natural ligand ACh (table 1). Replacement of Cfα1 by its *D. melanogaster* ortholog Dmα1 in *Xenopus* expression and electrophysiology experiments revealed that Dmα1/Ggβ2 was persistently activated by epibatidine, and was also highly sensitive to nicotinoid and neonicotinoid agonists, with EC_{50} values and orders of potency similar to that of Cfα1/Ggβ2 (table 2). Imidacloprid was the most potent agonist with an EC_{50} of 40 nM (table 2, Fig. 7C). Different to Cfα1/Ggβ2 were the Hill coefficients, which were for the neonicotinoids between 1.18 and 1.64, suggesting some positive cooperativity (table 2).

Ggα4/Ggβ2: the chicken neuronal receptor Ggα4/Ggβ2 expressed in *Xenopus* oocytes showed an ACh response with an EC_{50} of 11.5 μM and some positive cooperativity (n_H = 1.43), which is in line with the earlier report of Shimomura et al. (2004), while the nicotinoid nicotine gave an EC_{50} value of 760 nM (table 1). Epibatidine treatment led to persistent activation of Ggα4/Ggβ2 that could only insufficiently be washed off, which precluded EC_{50} determinations. Dose-response investigations of neonicotinoids on Ggα4/Ggβ2 in *Xenopus* oocytes showed that all 6 compounds investigated were partial agonists in the μM to mM range, with I_{max}[%ACh] values from only 0.82% (nitenpyram) to 64.9% (clothianidin). EC_{50} determinations showed that imidacloprid was 690-fold, nitenpyram 73-fold, acetamiprid 85-fold, dinotefuran 244-fold and clothianidin 142-fold less potent on the chicken receptor Ggα4/Ggβ2 than on Cfα1/Ggβ2, while loss of signal upon repeated exposure precluded EC_{50} determinations for thiacloprid (see table 1).

Table 1: *Xenopus* oocyte coexpression and electrophysiology of *C. felis* α1, *C. felis* α2 and chicken α4 with chicken β2: EC_{50}, Hill coefficient n_H and I_{max}

	Cfα1/Ggβ2			**Cfα2/Ggβ2**			**Gaα4/Ggβ2**		
	EC_{50} (μM) ±SD	**n_H ±SD**	**I_{max} [% ACh] ±SD**	**EC_{50} (μM) ±SD**	**n_H ±SD**	**I_{max} [% ACh] ±SD**	**EC_{50} (μM) ±SD**	**n_H ±SD**	**I_{max} [% ACh] ±SD**
ACh	0.05 +/- 0.01 (n=6)	1.03 +/- 0.10 (n=6)	100	9.0 +/- 1.5 (n=7)	2.20 +/- 0.22 (n=7)	100	11.5 +/- 3.5 (n=4)	1.43 +/- 0.07 (n=4)	100
Nic	0.02 +/- 0.00 (n=5)	1.33 +/- 0.15 (n=5)	37.2* +/- 5.7 (n=4)	2.58 +/- 0.46 (n=5)	1.68 +/- 0.23 (n=5)	63.8§ +/- 5.8 (n=6)	0.76 +/-0.13 (n=2)	1.1 +/- 0.1 (n=4)	84.2# +/- 2.6 (n=2)
Epi	n. d.	n. d.	n. d.	0.009 +/- 0.001 (n=4)	1.64 +/- 0.16 (n=4)	86.2§ +/- 7.5 (n=6)	n. d.	n. d.	n. d.
Imi	0.02 +/- 0.00 (n=5)	1.29 +/- 0.14 (n=5)	11.2* +/- 2.5 (n=5)	1.31 +/- 0.35 (n=6)	1.81 +/- 0.35 (n=6)	63.2§ +/- 4.2 (n=2)	13.8 +/- 1..0 (n=4)	1.13 +/- 0.04 (n=4)	24.8# +/- 9.05 (n=4)
Nit	0.63 +/- 0.22 (n=5)	1.26 +/- 0.14 (n=5)	44.7* +/- 9.0 (n=4)	24.4 +/- 6.4 (n=6)	2.12 +/- 0.19 (n=6)	43.5§ +/- 1.2 (n=2)	45.8 +/- 9.3 (n=4)	1.37 +/- 0.07 (n=4)	0.82# +/- 0.27 (n=3)
Acet	0.11 +/- 0.05 (n=5)	1.04 +/- 0.18 (n=5)	13.2* +/- 2.2 (n=5)	2.63 +/- 0.26 (n=4)	1.61 +/- 0.08 (n=4)	20§ +/- 3.9 (n=2)	9.4 +/- 0.91 (n=4)	1.22 +/- 0.06 (n=5)	5.01# +/- 0.52 (n=6)
Din	4.19 +/- 1.70 (n=5)	1.07 +/- 0.16 (n=5)	34.2* +/- 6.8 (n=5)	124.8 +/- 16.4 (n=5)	2.08 +/- 0.12 (n=5)	94.4§ +/- 23.1 (n=3)	1021.4 +/- 120.8 (n=5)	1.71 +/- 0.19 (n=5)	9.13# +/- 1.48 (n=5)
Clo	0.15 +/- 0.07 (n=5)	0.96 +/- 0.08 (n=5)	44.3* +/- 5.2 (n=4)	1.65 +/- 0.12 (n=4)	2.24 +/- 0.13 (n=4)	148§ +/- 0.6 (n=2)	21.3 +/-2.3 (n=3)	1.6 +/- 0.18 (n=4)	64.9# +/- 8.6 (n=6)

I_{max}[%ACh] was determined at the following saturating agonist concentrations: * nicotine 0.3 μM, imidacloprid 1 μM, nitenpyram 5 μM, acetamiprid 2.5 μM, dinotefuran 20 μM, clothianidin 30 μM. § nicotine 15 μM, epibatidine 0.1 μM, imidacloprid 6 μM, nitenpyram 100 μM, acetamiprid 15 μM, dinotefuran 400 μM, clothianidin 30 μM. # nicotine 10 μM, imidacloprid 200 μM, nitenpyram 700 μM, acetamiprid 100 μM, dinotefuran 7 mM, clothianidin 200 μM. n. d. : not determined. Standard deviations (SD) are indicated. The EC_{50} differences between Cfα1/Ggβ2 and Cfα2/Ggβ2 were analysed by Student's *t*-test for all agonists. The *p*-values for all pairs were below 0.0001.

Table 2: *Xenopus* oocyte coexpression and electrophysiology of *D. melanogaster* α1 and α2 with chicken β2: EC_{50}, Hill coefficient n_H and I_{max}

	Dmα1/Gaβ2			Dmα2/Gaβ2		
	EC_{50} (μM) ±SD	**n_H ±SD**	**I_{max} [% ACh] ±SD**	**EC_{50} (μM) ±SD**	**n_H ±SD**	**I_{max} [% ACh] ±SD**
ACh	0.07 +/-0.02 (n=8)	1.01 +/- 0.14 (n=8)	100	6.6 +/- 1.4 (n=5)	1.70 +/- 0.13 (n=5)	100
Epi	n. d.	n. d.	n. d.	0.003+/- 0,00 (n=5)	1.43 +/- 0,1 (n=5)	77.3§ +/- 6,4 (n=6)
Imi	0.04 +/- 0.01 (n=5)	1.25 +/- 0.14 (n=5)	9.5* +/- 0,7 (n=6)	0.84 +/- 0.17 (n=6)	1.53 +/- 0.14 (n=6)	70.8§ +/- 7.2 (n=5)
Nit	0.40 +/- 0.11 (n=6)	1.48 +/- 0.22 (n=6)	43.9* +/- 9.5 (n=6)	25.4 +/- 4.2 (n=5)	1.61 +/- 0.06 (n=5)	71.8§ +/- 11,1 (n=4)
Acet	0.23 +/- 0.08 (n=8)	1.41 +/- 0.18 (n=8)	16.1* +/- 2.2 (n=6)	2.0 +/- 0.2 (n=4)	1.63 +/- 0.17 (n=4)	54.4§ +/- 1.2 (n=3)
Din	4.56 +/- 1.06 (n=7)	1.64 +/- 0.26 (n=7)	39* +/- 0.8 (n=6)	105.7 +/- 8.5 (n=5)	1.94 +/- 0.21 (n=5)	101.2§ +/- 23.1 (n=3)
Clo	0.34 +/- 0.08 (n=8)	1.18 +/- 0.29 (n=8)	34.2* +/- 5 (n=6)	5.4 +/- 1.3 (n=6)	1.51 +/- 0.17 (n=6)	113.8§ +/- 9.6 (n=5)

I_{max}[%ACh] was determined at the following saturating agonist concentrations: * imidacloprid 1 µM, nitenpyram 5 µM, acetamiprid 2,5 µM, dinotefuran 20 µM, clothianidin 30 µM. § epibatidine 0,05 µM, imidacloprid 6 µM, nitenpyram 100 µM, acetamiprid 15 µM, dinotefuran 330 µM, clothianidin 30 µM. n. d. : not determined. Standard deviations (SD) are indicated. The EC_{50} differences between Dmα1/Ggβ2 and Dmα2/Ggβ2 were analysed by Student's *t*-test for all agonists. The *p*-values for all pairs were below 0.0001.

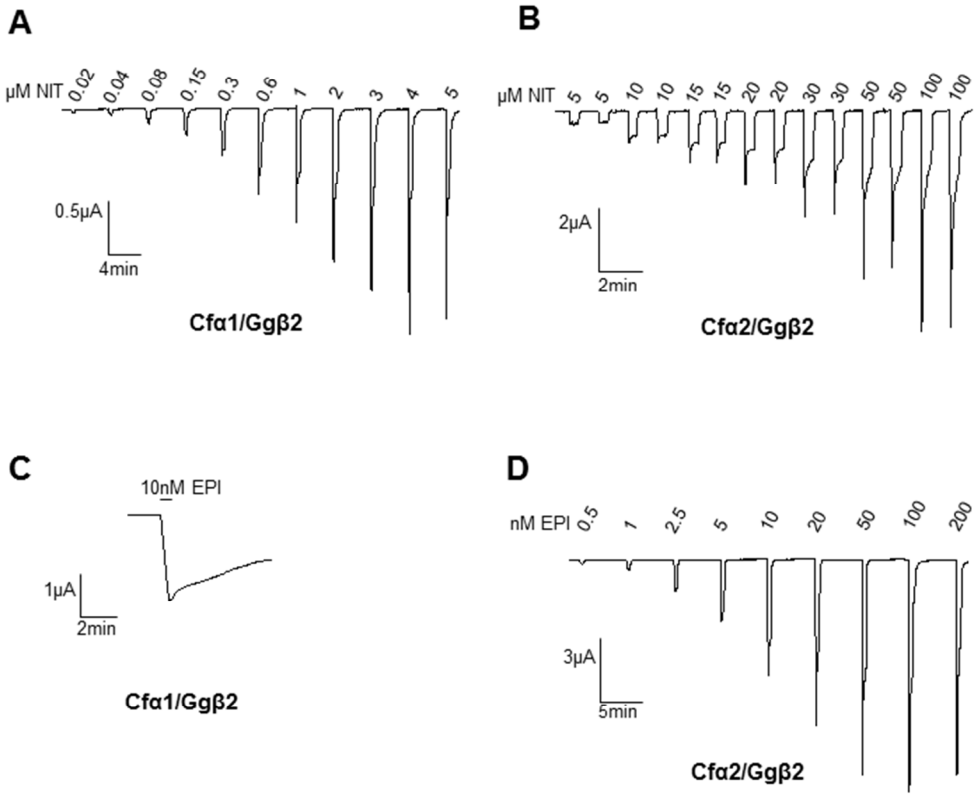

Figure 6: Current responses of nAChRs in *Xenopus* oocyte two electrode voltage clamp experiments – examples of nicotinoids and neonicotinoids. (A) Nitenpyram (NIT) titration of the current response of Cfα1. (B) Nitenpyram (NIT) titration of the current response of Cfα2. (C) Current response of Cfα1 to epibatidine (EPI). (D) Epibatidine (EPI) titration of the current response of Cfα2.

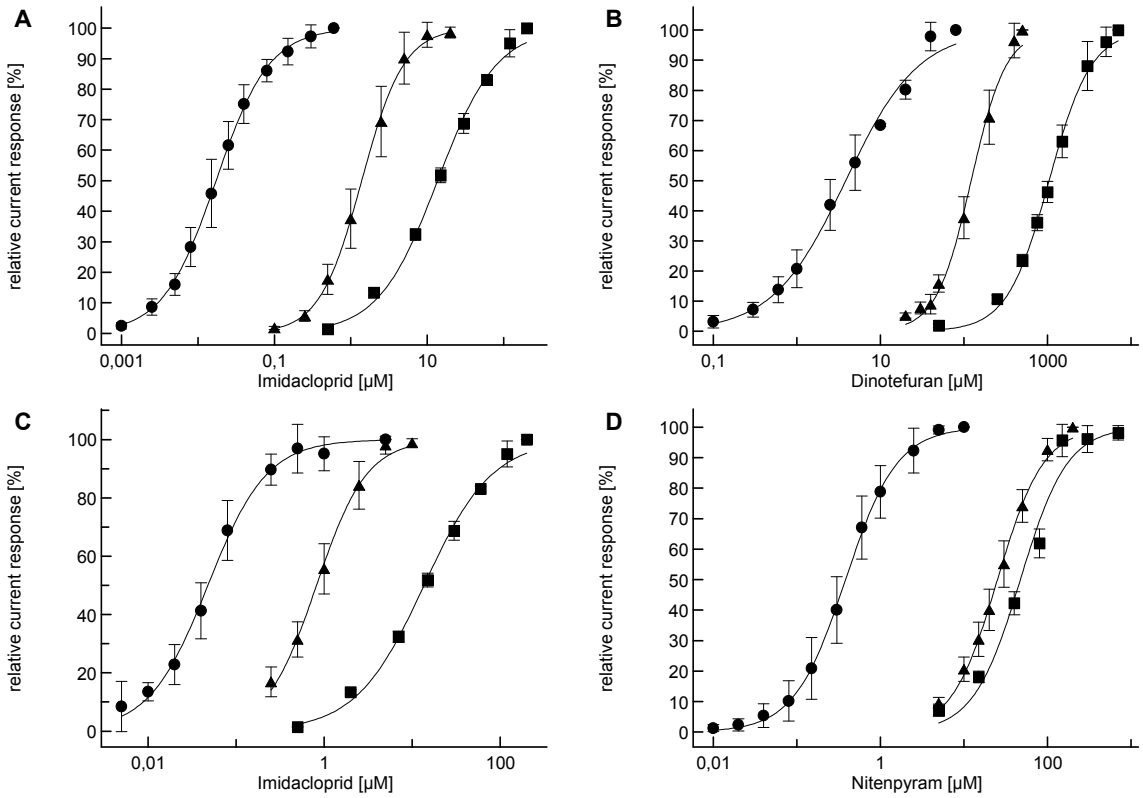

Figure 7: Hill curves of various receptor combinations with neonicotinoids as agonists: (A) Cfα1/Ggβ2 (●), Cfα2/Ggβ2 (▲), and Ggα4/Ggβ2 (■): titrations with imidacloprid; (B) (A) Cfα1/Ggβ2 (●), Cfα2/Ggβ2 (▲), and Ggα4/Ggβ2 (■): titrations with dinotefuran. (C) Dmα1/Ggβ2 (●), Dmα2/Ggβ2 (▲), and Ggα4/Ggβ2 (■): titrations with imidacloprid; (D) (A) Dmα1/Ggβ2 (●), Dmα2/Ggβ2 (▲), and Ggα4/Ggβ2 (■): titrations with nitenpyram. The error bars indicate the SD from 4-6 data points.

4. Discussion

In this study, we have isolated the full-length cDNAs encoding the nAChR subunits α1, α2 and α3 of the cat flea. In a previous study, the ligand binding domains of these three subunits were reported, and receptor chimera of Cfα1 and Cfα3 ligand binding domains with Dmα2 signal sequence, transmembrane helices and C-terminus were shown to form epibatidine and imidacloprid binding sites, when coexpressed with rat β2 in S2 cells (Bass et al. 2006). Here we have shown by *Xenopus* oocyte coexpression with the chicken β subunit Ggβ2 the electrophysiological functionality of all three full-length *C. felis* α subunit cDNAs. The ACh-evoked currents were in the μA range for Cfα1/Ggβ2 and Cfα2/Ggβ2, but in the low nA range for Cfα3/Ggβ2. The low currents precluded more detailed analysis of

the latter hybrid receptor by electrophysiological methods. We found that the combination Cfα2/Ggβ2 produces a hybrid receptor with an EC_{50} for its specific ligand ACh of 9 μM, a value similar to that determined for the chicken CNS receptor Ggα4/Ggβ2 (table 1). Cfα2/Ggβ2 is highly resistant to inhibition by α-bungarotoxin, as reported previously for the *D. melanogaster* ortholog (Bertrand et al. 1994). By contrast, the ACh EC_{50} of Cfα1/Ggβ2 was two orders of magnitude lower in the nM range, similar to that seen for Dmα1/Ggβ2 in this and in an earlier (Bertrand et al. 1994) study. Furthermore, the ion channel of this hybrid receptor proved to be sensitive to α-bungarotoxin block.

We first investigated the electrophysiology of Cfα2/Ggβ2 for two nicotinoids and 6 neonicotinoids, because in other insects hybrid receptors of the α2 subunits in combination with either rat or chicken β2 had been analysed as neonicotinoid binding sites and potential neonicotinoid targets by both electrophysiology and ligand binding experiments (Matsuda et al. 1998, Huang et al. 1999, Lansdell and Millar 2000, Nishiwaki et al. 2003, Ihara et al. 2003, Ihara et al. 2004, Shimomura et al. 2004, 2005, Tomizawa et al. 2005). In our studies, by far the most potent ligand for Cfα2/Ggβ2 was the analgetic alkaloid epibatidine (Badio and Daly 1994), acting as a full agonist with an EC_{50} of ~ 9 nM, whereas nicotine and all neonicotinoids were partial agonists showing EC_{50}s 2-4 orders of magnitude higher, in the μM range. Hereby, clothianidin acted as a superagonist, dinotefuran as full agonist, and all other neonicotinoids as well as nicotine as partial agonists of Cfα2/Ggβ2. While the EC_{50} of imidacloprid (1.31 μM, see Fig. 7A) on this potential neonicotinoid target was in an activity range that could perhaps be in agreement with its potent anti-flea activity, the potency of the other flea control market products nitenpyram (EC_{50} = 24.4 μM) or dinotefuran (EC_{50} = 124.8 μM, see Fig. 7B) appeared too low to explain their *in vivo* activity based on interaction with this receptor.

Investigation of nicotinoid and neonicotinoid agonist potency on Cfα1/Ggβ2 revealed that this hybrid receptor form is 10-100 times more sensitive to these ligands. Epibatidine activated this hybrid receptor persistently in the low nM range, while nicotine displayed an EC_{50} of 20 nM. All neonicotinoids investigated were partial agonists, that, except for dinotefuran (EC_{50} = 4.19 μM), showed EC_{50}s in the nM range, with imidacloprid with 20 nM being the most potent representative. In

earlier electrophysiology studies on *Drosophila* α subunits (Ihara et al. 2003), the combination Dmα1/Ggβ2 has been reported as being non-responsive to imidacloprid and poorly responsive to nitenpyram (EC_{50} = 10 μM). The discrepancy of these earlier results to those of our *C. felis* nAChR study was puzzling to us, especially given the fact that the amino acid sequences of the ligand binding domains of Cfα1 and Dmα1 are 96% identical. Therefore, we repeated and extended these earlier (Ihara et al. 2003) neonicotinoid *Xenopus* oocyte electrophysiology studies with hybrid receptors of both Dmα1 and Dmα2 (showing also 96% amino acid sequence identity to Cfα2 in the ligand binding domain) in combination with Ggβ2. In general in our study, the EC_{50} and I_{max}[%ACh] values for all ligands investigated and for both *D. melanogaster* hybrid nAChRs were highly similar to those obtained for the *C. felis* counterparts (compare table 1 with table 2). While for Dmα2/Ggβ2 our results for imidacloprid (EC_{50} = 0.84 μM, I_{max} = 71% *versus* the earlier results EC_{50} = 2.88 μM, I_{max} = 55%) and nitenpyram (EC_{50} = 25.4 μM, I_{max} = 72% *versus* the earlier results EC_{50} = 52.5 μM, I_{max} = 86%) were comparable to those of the previous study (Ihara et al. 2003), this was not the case for Dmα1/Ggβ2. In our hands, for *Xenopus* oocyte-expressed Dmα1/Ggβ2, imidacloprid was an extremely potent partial agonist (EC_{50} = 40 nM), followed by acetamiprid, clothianidin and nitenpyram, that also proved to be partial agonists with nM potencies (table 2, Fig. 7 C,D). We have no explanation for this discrepancy in results with respect to Dmα1/Ggβ2 between our study and that of Ihara et al. (2003), except that these authors used cDNA injection in a 1:1 ratio, while in our experiments cRNA 1:1 was employed. It is conceivable that cDNA injection leads to a different protein expression ratio of the two genes compared to cRNA injection, which may lead to distinct subunit stoichiometries that have been shown in other receptor systems to translate into different pharmacologies (Moroni et al. 2006). This possibility needs to be addressed in future experiments.

Our electrophysiology comparisons of the *C. felis* and *D. melanogaster* α subunits (see Fig. 7, tables 1 and 2) suggest that the insect α1 subunit may be a better candidate for being a crucial part of the *in vivo* neonicotinoid target than insect α2, which has been in the center of many previous investigations. This notion is supported by a number of earlier and current studies: Huang et al. (1999) reported ligand binding studies in S2 cells on hybrid receptors of *Myzus (M.) persicae* α1 and

α2 with rat β2, respectively. These authors found that while both hybrid receptors showed binding of epibatidine, only *M. persicae* α2/ rat β2 was a high affinity receptor for imidacloprid. Historic nomenclature can be deceiving, however: protein sequence alignments (see Fig. 3) reveal, that imidacloprid-binding *M. persicae* α2 belongs to the insect α1 family, while *M. persicae* α1, reportedly with no imidacloprid-binding capacity (Huang et al. 1999), is a member of the insect α2 family (Fig. 3). Target site-based neonicotinoid resistance has been reported for two different insect species, *N. lugens* (Liu et al. 2005) and *D. melanogaster* (Perry et al. 2008). In *N. lugens*, $Y^{151}S$ mutations have been identified in the nAChR subunits α1 and α3 that have been implicated in neonicotinoid resistance (Liu et al. 2005, 2006, 2009a,b; Yixi et al. 2009, Li et al. 2010). Mutagenesis studies in *D. melanogaster* led to the isolation of two neonicotinoid-resistant strains that had lesions in the Dmα1 and the Dmβ2 genes, respectively.

Taken together, the combined data of our current and earlier studies suggest that insect α1 may play a central role in neonicotinoid insecticide action. However, there should be awareness that the majority of studies were and still are performed with trans-species hybrid receptors, as 'insect-only' nAChRs have not yet been reported in heterologous systems (Millar 1999, 2009), with the exception of a homomeric locust receptor (Marshall et al. 1990, Amar et al. 1995). So far, it is unknown whether expression system factors, such as growth temperature (Lansdell et al. 1997), as yet unrecognized missing subunits, or the need for specific folding factors, such as RIC-3 (Millar 2008) or Lynx (Liu et al. 2009b), perhaps not present or insufficiently present in the current expression systems, are responsible for this failure. Therefore, potentially fundamental effects of insect-derived cosubunits other than the 'artificial' chicken or rat β2 on specificity, potency and maximal currents of recombinant insect nAChRs cannot be ruled out. There is already evidence that the use of a single insect α subunit in the current and most previously reported expression systems is too simplistic: in *D. melanogaster* biochemical data support the association of Dmα3 and Dmβ1 (Chamaon et al. 2000) as well as Dmα1, Dmα2 and Dmβ2 (Chamaon et al. 2002) in flies *in vivo*, and evidence for coassembly of Dmα1, Dmα2 and Ggβ2 has been obtained in heterologous expression experiments in *Xenopus* oocytes (Schulz et al. 2000). More recently, functional coassembly of *N.*

lugens α1 and α2 with rat β2 (Liu et al. 2009a) as well as of *N. lugens* α3 and α8 with rat β2 (Yixi et al. 2009) was observed in *Xenopus* oocyte expression experiments. These studies were followed by experiments that suggested an *in vivo* assembly of *N. lugens* subunits α1 and α2 and β1 and α3, α8 and β1 in this planthopper species (Li et al. 2010).

In summary, our hybrid receptor study provides evidence for a central role of the α1 subunit for neonicotinoid action in *C. felis* and *D. melanogaster*, and possibly insects in general. More definitive conclusions have to await the definition and heterologous functional expression of 'insect-only' nAChRs that have so far been elusive, despite more than 20 years of intense efforts.

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PAPER II

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Structure-activity relationships of acetylcholine derivatives with *Lucilia cuprina* nicotinic acetylcholine receptor α1 and α2 subunits in chicken β2 subunit hybrid receptors in comparison with chicken nicotinic acetylcholine receptor α4/β2.

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Abstract

Insect nicotinic acetylcholine (ACh) receptors (nAChRs) are the targets of several insecticide classes. In the present study, we report the gene identification and cloning of nAChR α1 and α2 subunits (Lcα1 and Lcα2) from the sheep blowfly *Lucilia cuprina*. *Xenopus* oocytes voltage clamp experiments as hybrids with the chicken β2 nAChR (Ggβ2) subunit resulted in ACh-gated ion channels with distinct dose–response curves for Lcα1/Ggβ2 (effective concentration 50% [EC_{50}] = 80 nM; n_H = 1.05), and Lcα2/Ggβ2 (EC_{50} = 5.37μM, n_H=1.46). The neonicotinoid imidacloprid was a potent agonist for the α-bungarotoxin-sensitive Lcα1/Ggβ2 (EC_{50} ~ 20 nM), while the α-bungarotoxin-resistant Lcα2/Ggβ2 showed a 30-fold lower sensitivity to this insecticide (EC_{50} = 0.62μM). Thirteen close derivatives of ACh were analysed in EC_{50}, Hill coefficient and maximum current (relative to ACh) determinations for Lcα1/Ggβ2 and Lcα2/Ggβ2 and the chicken Ggα4/Ggβ2 nAChRs, and comparisons relative to ACh allowed the definition of novel structure-activity and structure-selectivity relationships. In the case of *N*-ethyl-acetylcholine, the EC_{50} of the chicken Ggα4/Ggβ2 rose by a factor of 1000, while for both Lcα1/Ggβ2 and Lcα2/Ggβ2, potency remained unchanged. Further derivatives with insect nAChR selectivity potential were acetyl-α-methylcholine and trimethyl-(3-methoxy-3-oxopropyl)ammonium followed by acetylhomocholine and trimethyl-(4-oxopentyl) ammonium. Our results may provide guidance for the identification or design of insect-specific nAChR agonists using structure-based or *in silico* methods.

Introduction

The sheep blowfly *Lucilia* (*L.*) *cuprina* is the causative agent of major animal distress and economic losses in sheep husbandry in subtropical areas, such as Australia and South Africa. Disease is caused by the infestation of the living animal by *L. cuprina* larvae. This infestation is termed myiasis. Adult female flies lay eggs in the wool of sheep, particularly in humid parts affected by bacterial wool rot or by faecal and urine fleece soiling, a process called blowfly strike. Hatched larvae pass through three stages, initially feeding on nutrients within the fleece, then on epidermal tissue excised by secreted enzymes and by their mouthparts, and subsequently also on skin secretions provoked by this tissue damage (Hall & Wall, 1995; Tellam & Bowles, 1997). Treatment and control of this painful and debilitating disease is necessary, both for reasons of animal welfare and because of the considerable economic losses it causes to the sheep industry, which amount to up to AUS$260 million in Australia alone (Sackett & Holmes, 2006).

Practical blowfly strike control measures include general sheep management practices aimed at lowering strike frequency such as pizzle dropping, mulesing, tail docking or crutching (Tellam & Bowles, 1997) and the use of fly baits and traps (Urech et al., 2004; Broughan & Wall, 2006). Experimental approaches that have been pursued include selection of naturally resistant sheep (O'Meara et al., 1992; Tellam & Bowles, 1997), vaccination approaches against sheep blowfly larvae (Bowles et al., 1996; Tellam & Eisemann, 1998) and fly population control by sterile male release technology (Scott et al., 2004), but the main form of sheep myiasis control is the use of insecticides or insect growth regulators applied by dipping, jetting or dressing (Tellam & Bowles, 1997; Rothwell, 2005; Plant & Lewis, 2011).

Insecticide classes currently applied in sheep husbandry to combat blowfly strike include organophosphates, pyrethroids, avermectins (ivermectin) and spinosyns (spinosad) that bind to acetylcholinesterase, voltage-gated sodium channels, glutamate-gated chloride channels and nicotinic acetylcholine (ACh) receptors (nAChRs) as molecular targets, respectively. These lead to paralysis and death of the parasite. Among growth regulators, triazines (cyromazine) and pyrimidines (dicyclanil) are in use against *L. cuprina*. These compound classes are not directly harmful to the adult flies, but interfere with cuticle formation and lead to inhibition of hatching and moulting of larvae; however, resistance development is a

major concern in blowfly control. Resistance was first documented for organochlorine and carbamate insecticides, which led, amongst other factors, to their discontinuation as products, while the efficacy of organophosphates has been severely compromised by widespread resistance amongst *L. cuprina* populations (Levot, 1995). A dramatic example in the last decade is the emergence of resistance to the growth regulator diflubenzuron (Levot & Sales, 2004), which has rendered this compound largely ineffective against blowfly larvae in Australia.

The prime targets of insecticidal compounds are nAChRs. The fastest growing pesticide class, neonicotinoids (Gundelfinger, 1992; Millar & Denholm, 2007; Jeschke & Nauen, 2008), as well as spinosyns (Thompson et al., 2000; Sparks et al., 2001; Kirst, 2010) bind to these ion channels. The nAChRs are homo- or heteropentameric ligand-gated cation channels, whose domain structure is divided into an N-terminal Cys loop-containing ligand-binding extracellular domain, four transmembrane helices and a C-terminus facing the extracellular space, the hallmarks of the ligand-gated ion channel superfamily of proteins (Sine & Engel, 2006). nAChRs act in fast neurotransmission at cholinergic synapses in vertebrates (Corringer et al., 2000; Kalamida et al., 2007) and invertebrates (Jones & Sattelle, 2004; Jones et al., 2007). In contrast to mammals, where various nAChR subtypes occur in the muscles, the ganglia and the brain (Albuquerque et al., 2009; Millar & Gotti, 2009), the expression of functional nAChRs in insects appears to be restricted to the central nervous system (Sattelle, 1980; Breer & Sattelle, 1987).

Neonicotinoids act as partial, full and super-agonists of insect nAChRs and are broadly active and highly selective insecticides that are also used against cyclorrhaphan flies, such as fruit flies or nuisance flies (Nurita and Abu Hassan, 2010, Raga & Eidi Sato, 2011). The nAChR subunits α1, α2, and possibly also α3 and α8/β2 are implicated as potential molecular targets in various insects (Matsuda et al., 2001; Liu et al., 2005, 2006, 2009; Gao et al., 2007; Perry et al., 2008; Yixi et al., 2009; Li et al., 2010; Dederer et al., 2011), whereas spinosad appears to act on receptors containing nAChR subunit α6 (Perry et al., 2007; Baxter et al., 2010). While spinosad is an established blowfly insecticide (Levot et al., 2002), no compound of the neonicotinoid class is yet registered on the market for use against *L. cuprina*. Preliminary bioscreen experiments have shown that imidacloprid and nitenpyram have some efficacy in killing *L. cuprina* larvae (H. Williams and H. Zoller, MSD Animal

Health Innovation GmbH, unpubl. obs.), which prompted us to investigate the nAChR subunits of this important insect parasite as potential pesticide targets.

In the present study, we report the gene identification and molecular cloning of the two *L. cuprina* nAChR subunits Lcα1 and Lcα2. Using co-expression with chicken nAChR β2 in *Xenopus laevis* oocytes, the functionality of the gene product is shown and the pharmacology of standard nAChR agonists and antagonists established. Chemical synthesis of eight non-commercial and purchase of five additional ACh analogues allows a pharmacological comparison with the chicken nAChR α4/β2. The results establish a distinct structure-activity relationship for agonist action on the blowfly α subunits *vs.* the chicken α4 subunit, which may aid in the identification of fly-specific compounds.

Experimental procedures

Bacterial strains, plasmids, chemicals and insects

Bacterial cultures were grown in Luria-Bertani medium modified with supplements as required by the bacterial background and the introduced resistance genes. PCR products were cloned into pCR2.1-Topo, introduced into *Escherichia coli* Top10 cells (Invitrogen, Carlsbad, CA, USA) and, in some cases, subcloned into the mammalian expression vector pcDNA3.1(+) (Invitrogen). ACh, acetylthiocholine, nicotine and epibatidine were from Sigma (St Louis, MO, USA) and imidacloprid was from Dr. Ehrenstorfer. The *L. cuprina* flies used in this study were collected from long-term cultures maintained at Intervet Innovation GmbH, Schwabenheim, Germany, and displayed no known resistance to neonicotinoids, organophosphate or carbamate insecticides.

Identification, isolation and phylogenetic analysis of nicotinic acetylcholine receptor subunit genes from Lucilia cuprina *and chicken*

Total RNA was extracted from *L. cuprina* heads by a modification of the guanidinium thiocyanate/phenol extraction method (Chomczynski & Sacchi, 1987;

Trizol, Sigma). Other molecular biology techniques were performed essentially as described by Sambrook & Russell (2001). RT-PCR was performed using the Titan one tube RT-PCR system (Roche, Indianapolis, IN, USA) with total RNA (0.5–1 μg/50 μl) as template. In some cases, RT of total RNA was performed in a separate step followed by PCR under various conditions.

The chicken nAChR subunit genes α4 and β2 (*ggα4*, *ggβ2*) were described in Dederer et al., 2011. For the identification of nAChR genes of *L. cuprina*, degenerate primer pairs were constructed from the peptide sequences MKFGSW(S/T) and VEWD(L/M)(G/R)VPA, which are conserved in α1 and α2 subunits of many insect species (see also Fig. 1). The derived degenerate primer sequences were ATGAA(AG)TT(CT)GG(GATC)AG(CT)TGGAC and GC(GATC)GG(GATC)ACI(CT)(GCT)IA(AGT)(AGT)AT(AG)TCCCA(CT)TC. These primers were used in RT-PCRs with fly head total RNA as template. Two distinct RT-PCR products arising from *L. cuprina* NicRα1 and NicRα2 sequences of the expected size (seq1 and seq2, ATGAAATTTGGCTCTTGGACTTATGATGGTTACATGGTCGATTTAAGGCATCTTA AACAAACGCCAGATTCCGATAACATTGAAGTTGGCATTGACCTTCAGGACTATTA TATATCAGTTGAATGGGATATCATGCACCGTACCCGC and ATGAAATTTGGATCGTGGACCTATGATGGCGATCAAATTGATTTAAAACATATTA ATCAGAAAAATGACAAGGATAATAAAGTGGAAATTGGCATTGATTTACGTGAATA TTATCCAAGTGTGGAATGGGATATATTCACCGTGCCCGC, respectively) were identified and the missing cDNA sequences of the two *L. cuprina* genes were then obtained by nested rapid amplification of cDNA ends (RACE) using total RNA from fly heads as template and the SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) with generic flanking 5′- and 3′-RACE primer (Clontech), as outlined by the manufacturer. In the case of the *L. cuprina* NicRα1 gene, the 5′-gene region and the start codon was identified by 5′-RACE (first specific primer CTGATATATAATAGTCCTGAAGGTCAATGC, second specific primer GATGCCTTAAATCGACCATGTAACCATC), and the 3′-gene region and the stop codon by 3′-RACE (first specific primer GGACTTATGATGGTTACATGGTC, second specific primer CATTGAAGTTGGCATTGACCTTCAGG). For the identification of the *L. cuprina* NicRα2 5'-region, a simple RACE PCR was sufficient, while for the 3′-gene region, a nested RACE PCR approach was used (5′-RACE: specific primer

CCATTCCACACTTGGATAATATTCACG; 3'-RACE: first specific primer GTGGACCTATGATGGCGATCAAATTG, second specific primer GACAAGGATAATAAAGTGGAAATTGGC). Based on the deduced start and stop codon positions in the 5′- and 3′-RACE product sequences, the PCR primers CAGGTACCATGGGGAGCGTGCTGTTTGCAGCTG and GTGCGGCCGCCTATAAGTTCGTGTCGCTGCCCAT for *L. cuprina* nicotinic receptor α1 as well as CTGGTACCATGTCCGCCTTACACTACAAGTCGACAC and GAGCGGCCGCTTAGGATTTCTTTTCAGTTAGATTG for *L. cuprina* nicotinic receptor α2 were then designed for the RT-PCR amplification of the full length genes from fly head total RNA. The restriction enzyme sites introduced by the primers are underlined. The PCR products were cloned into pCR2.1-Topo and sequenced. ClustalW multiple sequence alignments of *L. cuprina* nAChR α subunits and other insect receptor subunits, the generation of phylogenetic trees and bootstrap analyses were performed with the DNAStar Lasergene software package. Bioinformatics analysis for the presence of ER import sequences and transmembrane helices were performed using SignalP3.0 (Bendtsen et al., 2004) (http://www.cbs.dtu.dk/services/SignalP/) and TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/), respectively.

Xenopus laevis *oocyte expression and electrophysiology experiments with nicotinic acetylcholine receptor subunit genes from* Lucilia cuprina *and chicken*

The ORFs of Ggα4, Ggβ2 (both from chicken, Dederer et al., 2011), Lcα1 and Lcα2 were subcloned into pcDNA3.1(+) downstream of the T7 promoter *via* restriction enzyme sites introduced by the PCR primers. The resulting plasmids were linearized by *Spe*I (Lcα1), *Hind*III (Lcα2), *Eco*RI (Ggβ2) or *Xba*I (Ggα4), and *in vitro* transcription to obtain 5′-capped cRNA, and subsequent polyadenylation was performed using the mMESSAGE mMACHINE T7 transcription kit (Ambion, Austin, TX, USA) according to the manufacturers protocol. Transcripts were recovered by LiCl precipitation, dissolved in nuclease-free water at a final concentration of ~ 2 μg/μl, and stored at -80 °C until use.

Defolliculated *Xenopus laevis* oocytes (sates V-VI) were purchased from Ecocyte Biosciences (Castrop-Rauxel, Germany). 50.6 nl cRNAs were injected in a

1:1 molar ratio using a micromanipulator (World Precision Instruments, Berlin, Germany). The oocytes were incubated for 48–96 h at 17 °C in modified Barths solution (5 mM Hepes pH 7.2, 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 2.4 mM Na-pyruvate, PS). Oocytes held in bath were perfused with Barths solution (5 mM Hepes pH 7.2, 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$) at a flow rate of ~1.8 ml/min were voltage clamped at –60 mV using the two electrode clamp mode of an Turbo Tec-03x amplifier (NPI Electronic, Tamm, Germany). Electrodes were pulled from borosilicate glass (Science Products, Hofheim, Germany) using the Puller PC-10 (Narishige Group, Tokyo, Japan), and filled with 3 M KCl. The electrode resistance ranged between 1–5 MΩ on the current-passing side. Agonist solutions, freshly prepared in Barths solution from dimethylsulfoxide- (DMSO-) stock solutions (100 mM – 1 M), with final DMSO concentrations not exceeding 1,5% (v/v), were applied *via* bath perfusion for 30 sec. The resulting inward current was recorded using CellWorksLite 5.5.1 (NPI Electronic), and analysed later. An interval of 2 min was routinely maintained between agonist applications, which was elongated in some case to up to 5 min. Dose–response curves were analysed using the Hill equation ($I = I_{max}([A]^{nH}/[A]^{nH} + EC_{50}{}^{nH})$, where "I" represents the current response measured at its peak, "I_{max}" the maximum response in the experiment, "EC_{50}" the agonist concentration for 50% maximum response, and "n_H" the Hill coefficient. Calculations of these parameters as well as standard deviations were carried out using XL-fit (Microsoft®Excel™). For I_{max}[% ACh] determinations of nicotinoid and neonicotinoid agonists on different nAChR subunit combinations, a saturating ACh concentration was applied first, the maximum current recorded, and then saturating test agonist concentrations were applied. The percentage of maximum current (I_{max}[% ACh]) relative to ACh was calculated.

Synthesis of acetylcholine analogues

General information: All commercially available chemicals were purchased from Sigma-Aldrich, except iodoethane and trimethylamine (Fluka, Buchs, Switzerland), sodium carbonate (Merck, Darmstadt, Germany) and (±)-2-dimethylamino-1-propanol (ACC Corp., San Diego, USA). If not indicated the purity of chemicals was ≥98%. Anhydrous diethylether, acetone and ethanol were purchased from Acros Organics (Geel, Belgium). ^{1}H-NMR spectra were obtained on a Bruker

DPX300 (300.13 MHz) spectrometer. Data are reported as chemical shifts (δ ppm) internally referenced to the solvent (D_2O unless otherwise noted) and splitting patterns (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet). The purity and content of the synthesized products were determined using ^{1}H-NMR quantification with maleic acid as internal reference. Water content of synthesized products was determined by Karl Fisher titration and found to be below 1% for all synthesized products. Syntheses were generally performed under an argon atmosphere.

- 3-Acetoxypropyl(trimethyl)ammonium (acetylhomocholine) iodide (**11**)

As outlined by Yamada et al. (1987) the synthesis was carried out according to the method of Renshaw et al. (1938) with some modifications. To a stirred solution of 3-dimethylamino-1-propanol (1.2 ml, 10 mmol) in 50 ml anhydrous ether, acetyl chloride (0.7 ml, 10.2 mmol) was added drop-wise with ice cooling. Subsequently, the suspension was stirred for 20 h at room temperature. The resulting solid reaction product was isolated by filtration, dissolved in an aqueous 10 % sodium carbonate solution and extracted with ether. The ether extract was dried with $MgSO_4$, filtered and the solvent was removed under reduced pressure. The resulting residue was stirred with 20 % molar excess of methyl iodide (0.7 ml, 11.1 mmol) in 50 ml anhydrous ether for 20 h at room temperature. The reaction mixture was filtered, the solid residue was washed with ether and dried under reduced pressure to yield 1.9 g (65% yield) of a solid. ^{1}H-NMR δ 4.2 (t, 2H), 3.5 (m, 2H), 3.2 (s, 9H), 2.2 (m, 2H), 2.1 (s, 3H). Content: 99.3% ± 0.4%.

The compounds (**2**) (**3**) and (**5**) were synthesized according to the procedure for the synthesis of (**11**):

- (2-Acetoxy-1-methyl-ethyl)-trimethyl ammonium (acetyl-α-methylcholine) iodide (**5**)

Starting material: (±)-2-dimethylamino-1-propanol (1 g, 10 mmol), acetyl chloride (0.7 ml, 10.2 mmol), methyl iodide (0.6 ml, 8.8 mmol). Product obtained as solid: 0.5 g (18% yield). ^{1}H-NMR δ 4.5 (m, 2H), 3.9 (m, 1H), 3.2 (s, 9H), 2.2 (s, 3H), 1.5 (m, 3H). Content: 93.4% ± 0.8%.

- 2-Acetoxyethyl-dimethyl-propyl-ammonium (acetyl-N-propylcholine) iodide (**3**)

Starting material: 2-dimethylaminoethanol (5 ml, 50 mmol), acetyl chloride (3.7 ml, 51 mmol), propyl iodide (5.2 ml, 53.8 mmol). Product obtained as solid: 1 g (7% yield). ^{1}H-NMR δ 4.5 (m, 2H), 3.7 (m, 2H), 3.4 (m, 2H), 3.2 (s, 6H), 2.2 (s, 3H), 1.8 (m, 2H), 1.0 (t, 3H). Content: 94.3% ± 0.3%.

- 2-Acetoxyethyl-dimethyl-ethyl-ammonium (acetyl-N-ethylcholine) iodide (**2**)

Starting material: 2-dimethylaminoethanol (5 ml, 50 mmol), acetyl chloride (3.7 ml, 51 mmol), ethyl iodide (4.4 ml, 54.9 mmol). Product obtained as solid: 3.2 g (22% yield). ^{1}H-NMR δ 4.5 (m, 2H), 3.7 (m, 2H), 3.5 (q, 2H), 3.2 (s, 6H), 2.2 (s, 3H), 1.4 (t, 3H). Content: 99.3% ± 1.7%.

- Trimethyl-(3-methoxy-3-oxopropyl) ammonium iodide (**14**)

Methyl-3-(dimethylamino)-propionate (0.7 ml, 5 mmol) was stirred with methyl iodide (0.4 ml, 6 mmol) in 50 ml anhydrous ether for 20 h at room temperature. The product was isolated by filtration of the reaction mixture and drying under reduced pressure to yield 0.7 g of a solid (26% yield). ^{1}H-NMR δ 3.8 (s, 3H), 3.7 (t, 2H), 3.2 (s, 9H), 3.0 (t, 2H). Content: 97.2% ± 0.7%.

- Trimethyl-(2-ethoxyethyl) ammonium bromide (**7**)

To a stirred solution of trimethylamine (6.2 ml, 26 mmol; 4.2 M in ethanol), bromo-2-ethoxy ethane (2.5 ml, 20 mmol) was added drop-wise with ice-cooling. The solution was allowed to stir for 3 days at room temperature and subsequently diluted with isopropanol (10 ml). The resulting solid reaction product was isolated by filtration and washed with isopropanol (2 x 15 ml) and dried under reduced pressure to yield 2.55 g (72% yield) of a solid. ^{1}H-NMR (CD_3OD) δ 3.9 (m, 2H), 3.7 (m, 2H), 3.6 (q, 2H), 3.3 (s, 9H), 1.3 (t, 3H). Content: 94.5% ± 4.3%

- Trimethyl-(pentyl) ammonium iodide (**8**)

The synthesis was carried out according to the procedure of Menger & Venkataram (1986) with some modifications. Pentyl iodide (0.7 ml, 5.1 mmol) was treated with trimethylamine (2.4 ml, 10 mmol; 4.2 M in ethanol) at room temperature for 20 h in a sealed pressure tube. The reaction mixture was poured into 50 ml of ether, stirred, filtered and washed with ether to give 1.4 g (98% yield) of a solid. ^{1}H-NMR δ 3.3 (m, 2H), 3.1 (s, 9H), 1.8 (m, 2H), 1.4 (m, 4H), 0.9 (t, 3H). Content: 101.5% ± 2.0%.

- Trimethyl-(4-oxopentyl) ammonium iodide (**6**)

The Finkelstein reaction was carried out according to the procedure of Chiarello & Joullie (1988). 5-Chloro-2-pentanone (1.2 ml, 10 mmol) and sodium iodide (15 g, 100 mmol) in 50 ml of acetone were allowed to reflux for 20 h. The reaction mixture was cooled under stirring to prevent clotting of precipitated salts, diluted with 100 ml of ether and filtered. The filtrate was washed with saturated NaCl solution and 20% $Na_2S_2O_3$ solution, dried over $MgSO_4$, filtered and evaporated to dryness under reduced pressure. The resulting 5-iodo-2-pentanone (2.1 g, 9.8 mmol) was used for

the subsequent reaction with trimethylamine (3.6 ml, 15 mmol; 4.2 M in ethanol) as described for product (**8**). Product obtained as solid: 1.1 g (42% yield). ^{1}H-NMR δ 3.3 (m, 2H), 3.1 (s, 9H), 2.7 (t, 2H), 2.2 (s, 3H), 2.0 (m, 2H). Content: 91.5% ± 0.8%.

Results

Identification of Lucilia cuprina *genes encoding for nicotinic acetylcholine receptor subunits, cDNA isolation and bioinformatics analysis*

For the identification of nAChR α1 and α2 subunit genes of *L. cuprina*, degenerate primer pairs were deduced from conserved peptide sequences (Fig. 1). Degenerate primer reverse transcription (RT)-PCR was performed using total RNA isolated from *L. cuprina* heads. RT-PCR products of the expected size (~150 bp) were cloned and sequenced. Two different types of sequences were identified (*seq1 and seq 2, see* Experimental procedures). The deduced protein sequences of the degenerate primer RT-PCR products showed extensive sequence identities to the corresponding regions of the *Drosophila melanogaster* α1 and α2 (Dmα1 and Dmα2, respectively) subunit genes (NM_79757/X53583). The peptide sequence from *seq1* was slightly closer to Dmα1 than to Dmα2, while the translated *seq2* was slightly more homologous to Dmα2. The missing cDNA sequences of the corresponding *L. cuprina* nicotinic receptor α1 and α2 genes were obtained by 5'- and 3'-RACE using fly head total RNA as template. Based on the deduced start and stop codon positions in the 5'- and 3'-RACE product sequences of *L. cuprina* nicotinic receptor α1 (accession numbers KC134196 and KC134197, respectively), and nicotinic receptor α2 (accession numbers KC134199 and KC134200, respectively), PCR primers were then designed for the RT-PCR amplification of the respective full length genes from total RNA. In the case of *L. cuprina* nicotinic receptor α1, an RT-PCR product with a 1689 bp open reading frame [ORF (*lca1*)] was identified (accession number KC134198), while for *L. cuprina* nicotinic receptor α2, the amplicon comprised an ORF of 1692 bp (*lcα2*; accession number KC134201). Consensus sequences devoid of PCR errors were identified by the sequencing of four and five independent

plasmid-cloned PCR fragments in the case of *lcα1* and *lcα2*, respectively, and by direct PCR product sequencing.

The deduced polypeptide sequence of these new *L. cuprina* genes, *lcα1 and lcα2*, showed some sequence identity with each other (57.6%) and strong sequence identity with corresponding nicotinic receptor α1 and α2 subunits from other insect species, particularly *D. melanogaster* α1 (ALS, NM_079757) and α2 (SAD, X53583) with 92.9 and 90.6%, respectively, and *Ctenocephalides felis* (cat flea) α1 (FR689743) and α2 (FR689746) subunits (Fig. 1) with 80.1 and 83.5%, respectively. To a lesser degree there was homology to α2 (X81888) and α1 (X81887) subunits of the hemimetabolous insect *Myzus persicae*, with peptide sequence identities of 71.3 and 69.4%, respectively. Construction of a DNAStar/ClustalW-based molecular tree (Fig. 2) confirmed that the *lcα1* and *lcα2* genes belong to the insect nicotinic receptor subunit α1 and α2 families, respectively.

Inspection of the sequence of the Lcα1 and Lcα2 polypeptides with bioinformatics tools revealed within the predicted extracellular domains the presence of the cysteine loop and the loop A-C motifs typical for nicotinic receptor α subunit ligand-binding sites (Arias, 1997; Corringer et al., 2000; Albuquerque et al., 2009; Fig. 1). These loops contained a number of highly conserved amino acid residues: within the cysteine loop the two cysteines corresponding to C^{128} and C^{142} in the reference Torpedo α subunit; in loop A the amino acid residues corresponding to tryptophane W^{86} and tyrosine Y^{93}; in loop B residues corresponding to W^{149} and Y^{151}; and within loop C amino acids corresponding to C^{192} and C^{193} as well as Y^{190} and Y^{198} (all Torpedo α subunit numbering; Arias, 1997; Corringer et al., 2000; Albuquerque et al., 2009, Fig. 1). Kyte–Doolittle hydrophobicity plots of Lcα1 and Lcα2 suggested the presence of N-terminal endoplasmic reticulum (ER) import signal sequences and of several transmembrane regions in all three polypeptide sequences (not shown). Analysis of the two *L. cuprina* nicotinic receptor α subunit sequences with Signal P4.0 showed an ER import signal sequence probability of 0.848 for Lcα1, with a predicted cleavage site between A^{21} and N^{22}, and for Lcα2 a probability of 0.930 with a predicted cleavage site between A^{32} and N^{33} (Fig. 1). Further analysis with the transmembrane helix detection program TMHMM predicted in both gene products the four transmembrane helices typical for nAChR subunits and ligand-

gated ion channels (LGIC) in general (Fig. 1 and data not shown; Arias, 1997; Corringer et al., 2000; Albuquerque et al., 2009). Within and neighbouring the second transmembrane helices TM2, the highly conserved amino acid residues of the nAChR ion channel 'rings' corresponding to D^{234}, E^{237}, S^{240}, T^{244}, L^{247}, V^{251}, L^{254} and E^{258} (chicken α7 subunit numbering, Corringer et al., 2000) are either also present in Lcα1 and Lcα2, or replaced by structurally and/or functionally similar amino acids (Fig. 1; S^{240} → A, Lcα2; V^{251} → M, Lcα2, T^{244} → S, Lcα1 and Lcα2; chicken α7 numbering, Corringer et al., 2000). A search for potential N-glycosylation sites using NetNGlyc 1.0 revealed two fully conserved consensus sequences with high scores in the algorithm in Lcα1 and Lcα2 at the amino acid positions 45 and 233, and at the positions 56 and 245, respectively. As they are located in the putative extracellular domains of the receptor subunits, it appears likely that they are occupied by N-glycans in the mature receptor protein. In Lcα2 three further potential N-glycosylation motifs were identified at positions 389, 407 and 557; however, the TMHMM algorithm predicted that the motifs at 389 and 407 are located within an intracellular loop and are therefore most likely not modified by the N-glycosylation machinery in the ER. The motif at 557 was predicted to be in the short extracellular C-terminal tail of the receptor. It is also present in Dmα2 and may carry an N-glycan in the mature protein.

Functional characterization of Lcα1 and Lcα2.

Co-expression of Lcα1 and Lcα2 as hybrid receptors with chicken β2 (Ggβ2, Bertrand et al., 1994) resulted in functional receptors that responded to acetylcholine by dose-dependent currents (Fig. 3A,B). Generally, expression of Lcα2/Ggβ2 receptors led to stronger currents (range 5–20 μA) upon acetylcholine stimulation compared with Lcα1/Ggβ2 (range 1–2 μA). By contrast, with respect to the effective concentration 50% (EC_{50}), Lcα1/Ggβ2 proved to be much more responsive to acetylcholine-mediated channel opening (EC_{50} = 80 nM, Table 1) than Lcα2/Ggβ2 (EC_{50} = 5.37 μM, Table 1). Lcα1/Ggβ2 was sensitive to α-bungarotoxin block of acetylcholine agonist action, while Lcα2/Ggβ2 was unaffected by this snake toxin (Fig. 3C). Nicotine was a partial agonist of Lcα1/Ggβ2 [maximum response in the experiment (I_{max}) ~ 11.6%] with an EC_{50} of ~ 20 nM, while this tobacco alkaloid was a full agonist of Lcα2/Ggβ2 (I_{max} ~ 100%), but with much lower potency (EC_{50} ~ 940

nM, Table 1). Since repeated nicotine application resulted in pronounced receptor run-down of both Lcα1/Ggβ2 and Lcα2/Ggβ2 that could not be reversed by washing (data not shown, and Fig. 6C), the EC_{50} and I_{max} values have to remain tentative and Hill coefficients were not determined. The tree frog alkaloid epibatidine (Badio & Daly, 1994) led to irreversible channel opening in the case of Lcα1/Ggβ2 (Fig. 3D), while with Lcα2/Ggβ2 (Fig. 3E), it acted as an extremely potent full agonist whose repeated application led to receptor run-down (data not shown) (tentative EC_{50} = 7 nM, tentative I_{max} = 100%; Table 1, Fig. 3E). The neonicotinoid imidacloprid was a highly potent partial agonist of Lcα1/Ggβ2, which gave rise however to only small currents (tentative EC_{50} = 20 nM, I_{max} = 5.1%). For Lcα2/Ggβ2, this insecticide was a full agonist (I_{max} = 104%) with some cooperativity (n_H = 1.52) and an EC_{50} of 620 nM (Table 1, Fig. 5F).

Chemical compounds for structure–activity relationships of acetylcholine backbone derivatives: purchase, synthesis and analytics

To obtain a more detailed picture about the structural requirements for agonist action on Lcα1 and Lcα2-containing nAChRs, a collection of 13 close derivatives of the natural ligand acetylcholine was assembled (structures **1**–**14**, Fig. 4B). Acetylcholine and five derivatives (**1**, **4**, **9**, **10**, **12**, **13**) could be purchased, while eight further compounds (**2**, **3**, **5**, **6**, **7**, **8**, **11**, **14**) were synthesized in the present study as their bromide or iodide salts. The compound identity was confirmed by ^{1}H-NMR. Purity and content of the synthesis products were assessed by ^{1}H-NMR quantification and were uniformly >90%.

Five centres for derivatization of acetylcholine were chosen (R^1, R^2, R^3, X, R^4; Fig. 4A): first, one methyl group (R^1) of the quaternary ammonium group was replaced by ethyl (**2**) and n-propyl (**3**). The ethylene group was substituted by methyl either in the α- (R^2) or in the β-position (R^3) yielding *rac*-α-methylcholine (**5**) and *rac*-β-methylcholine (**4**), respectively. The choline oxygen (X) experienced the most modifications: replacement of the oxygen by sulphur led to acetylthiocholine (**9**), while replacement by a methylene group yielded trimethyl-(4-oxopentyl)-ammonium (**6**). Homologation of the ethylene group by one carbon led to acetylhomocholine


Consensus
Loop A
Cys Loop
Loop B
Loop C
TM1
TM2
TM3
TM4
Mp-a1.pro
Mp-a2.pro
Dm-ALS.pro
Dm-SAD.pro
Lc-a1.pro
Lc-a2.pro

Figure 1. CLUSTALW amino acid sequence alignment of *Lucilia cuprina* nicotinic acetylcholine (ACh) receptors (nAChR) α subunits. The predicted endoplasmic reticulum (ER) import signal sequences are highlighted by grey shading of black letters, and the predicted cleavage site is indicated by a grey triangle. The conserved tyrosine and tryptophane residues within the loop structures are marked with filled triangles, while the half-cystines of the Cys loops and the ligand binding sites are highlighted by connected open circles. The conserved amino acid residues forming the nAChR ion channel 'ring' in and near TM2 (Corringer et al., 2000) are highlighted by black circles (full conservation) or grey circles (partial conservation). The two conserved N-glycosylation sites in the extracellular domain (NG) are shown under the consensus sequence and are highlighted by light grey shading of white letters. The conserved extracellular loop structures (loop A, Cys loop, loop B, loop C) and the four predicted transmembrane helices (TM1–TM4) are highlighted by black bars under the consensus sequence. The black arrows indicate the conserved NicRα subunit peptide sequences used for the construction of degenerate PCR primers.

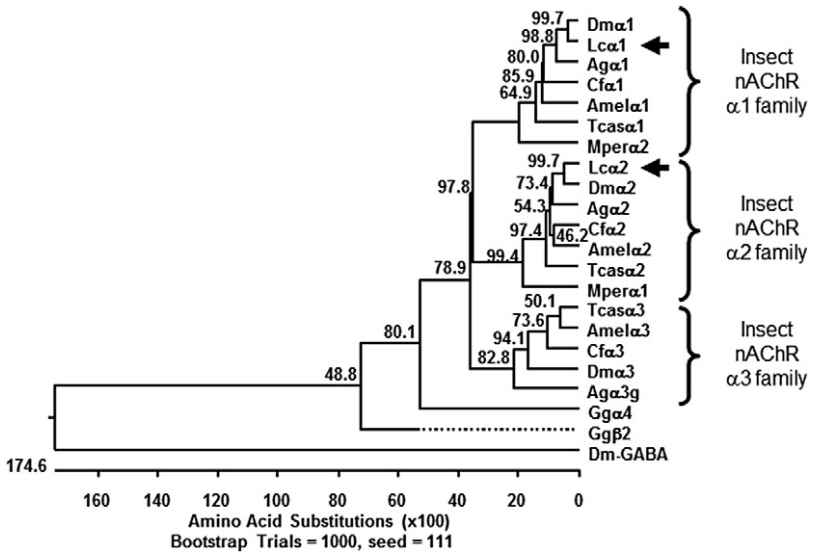

Figure 2. Amino acid sequence identity relationships of Lcα1 and Lcα2 to other insect nicotinic acetylcholine receptor subunits: phylogenetic dendrograms. The dendrogram (DNAStar) was derived from CLUSTALW-aligned protein sequences of Lcα1 (KC134198) and Lcα2 (KC134201) identified in this study (highlighted by black arrows) together with the *Drosophila melanogaster* α subunits 1–3 (Dmα1-α3, accession No: NM_079757, X53583, Y15593, respectively) as well as the α1–3 sequences of *Anopheles gambiae* (Agα1-α3; accession No: AY705394, AY705395, AY705396, respectively), of *Apis mellifera* (Amelα1-α3; accession No: DQ026031, NM_001011625, DQ026032, respectively), of *Tribolium castaneum* (Tcas-α1-α3; accession No: EF526080, EF526081, EF526082, respectively) and of *Ctenocephalides felis* (Cfα1-α3; accession No: FR689743, FR689746, FR689749, respectively) was constructed using DNAStar. In addition, the α1–2 sequences from *Myzus persicae* (Mperα1-α2, accession No: X81887 and X81888, respectively), and, for reference, *Gallus gallus* (chicken) α4 and β2 (Ggα4, Ggβ2; accession No: AJ250361 and AJ250362, respectively) were included. *D. melanogaster* GABA-gated chloride channel (Dm-GABA; accession No: M69057) served as an outgroup.

(**11**), while replacement of the acetyl group by -H or by $-PO_3^{2-}$ led to choline (**12**) and phosphocholine (**13**), respectively.Inversion of the ester moiety in acetylcholine led to its corresponding methyl ester (**14**). Removal of the carbonyl oxygen in acetylcholine (X = CO → X = CH_2, Fig 4A) led to the choline ethyl ether (**7**), while simultaneous replacement of the ether oxygen by methylene resulted in trimethyl-pentyl ammonium (**8**). Replacing one methyl group in acetylcholine by amino (R^4 = $COCH_3$ → R^4 = $CONH_2$, Fig 4A) led to carbamoylcholine (**10**). Taken together, these 13 compounds represent a comprehensive set of acetylcholine derivatives for structure-activity relationship studies of agonist potencies on nAChRs.

Structure–activity and structure–selectivity relationships of acetylcholine backbone analogues on Xenopus *oocyte-expressed Lcα1/Ggβ2, Lcα2/Ggβ2 and Ggα4/Ggβ2 nicotinic acetylcholine receptors*

Derivatives with modification of R^1: investigation of the quarternary amine. Replacement of one N-methyl group by ethyl in acetylcholine (compound (**2**), Fig. 4B) led in *Xenopus* oocyte voltage clamp experiments to EC_{50} values equal to or even lower than those for acetylcholine in the case of Lcα1/Ggβ2 and Lcα2/Ggβ2 nAChR, respectively. By contrast, in the case of the chicken ganglionic nAChR Ggα4/Ggβ2, the EC_{50} rose by a remarkable three orders of magnitude (factor 964) for acetyl-N-ethylcholine (**2**) relative to acetylcholine (Tables 1 and 2, Fig. 5A, B). I_{max} determinations showed that acetyl-*N*-ethylcholine (**2**) behaves as a non-cooperative partial agonist in the case of Lcα1/Ggβ2, as a full agonist with some cooperativity in the case of Lcα2/Ggβ2, and as a non-cooperative superagonist for Ggα4/Ggβ2 (Tables 1 and 2). In the case of acetyl-*N*-propylcholine (**3**), the EC_{50} values rose moderately relative to the acetylcholine EC_{50} values for both Lcα1/Ggβ2 and Lcα2/Ggβ2, where this derivative behaved as a partial agonist with noncooperative and partially cooperative curve characteristics, respectively (Tables 1 and 2). In the case of chicken Ggα4/Ggβ2, the EC_{50} of this noncooperative partial agonist rose by two orders of magnitude (factor 101) relative to acetylcholine.

Derivatives with modification of R^2: investigation of backbone methyl substitutions. Substitution of the acetylcholine methylene group next to the choline oxygen by a methyl group [acetyl-β-methylcholine, (**4**)] led to strong increases in EC_{50} for both receptors Lcα1/Ggβ2 and Ggα4/Ggβ2, by factors of 287 and 830, respectively. In the case of Lcα1/Ggβ2, acetyl-β-methylcholine (**4**) acted as a weakly cooperative partial agonist, while for the chicken nAChR Ggα4/Ggβ2, this compound was a full agonist displaying some cooperativity (Tables 1 and 2). In the case of Lcα2/Ggβ2, a marked receptor run-down of the ion channel function was noticed (Fig. 6A) for the weak partial agonist (**4**) (Table 2). The densitization could not be reversed by prolonged washing (Fig. 6A); therefore, EC_{50} determinations could only be performed tentatively and yielded a value of ~ 455 μM, some 85-fold higher than for acetylcholine (Tables 1 and 2). For the isomeric acetyl-α-methylcholine (**5**), very different results were observed: for both insect α-subunit-containing nAChR (Lcα1/Ggβ2, Lcα2/Ggβ2), the EC_{50} values remained in the same range as for their natural ligand acetylcholine,

while in the case of the chicken nAChR Ggα4/Ggβ2, the EC_{50} rose by a factor of 427 relative to acetylcholine (Tables 1 and 2, Fig. 5A and 5C). This compound acted as a noncooperative partial agonist, a partially cooperative full agonist and a negatively cooperative superagonist with Lcα1/Ggβ2, Lcα2/Ggβ2 and Ggα4/Ggβ2, respectively.

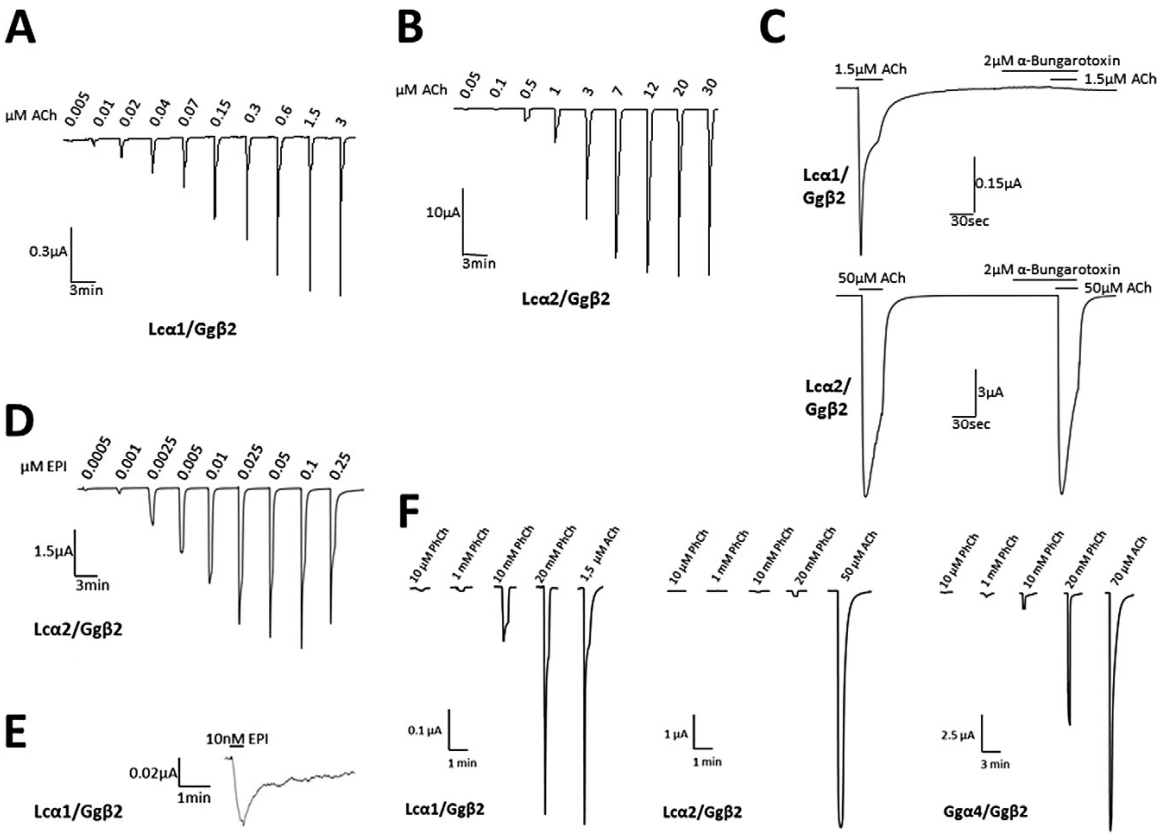

Figure 3. Electrical current responses of heteromeric nicotinic acetylcholine (ACh) receptors (nAChRs) with Lcα1 or Lcα2 or Ggα4 and Ggβ2 subunits. (A) ACh dose response of Lcα1/Ggβ2; (B) ACh dose response of Lcα2/Ggβ2; (C) α-bungarotoxin sensitivity of the ACh responses of Lcα1/Ggβ2 and Lcα2/Ggβ2; (D) Epibatidine dose response of Lcα2/Ggβ2; (E) Current response of Lcα1/Ggβ2 to epibatidine (EPI) (**16**); (F) Current responses of Lcα1/ Ggβ2, Lcα2/Ggβ2 and Ggα4/Ggβ2 to phosphocholine (PhCh) (**13**).

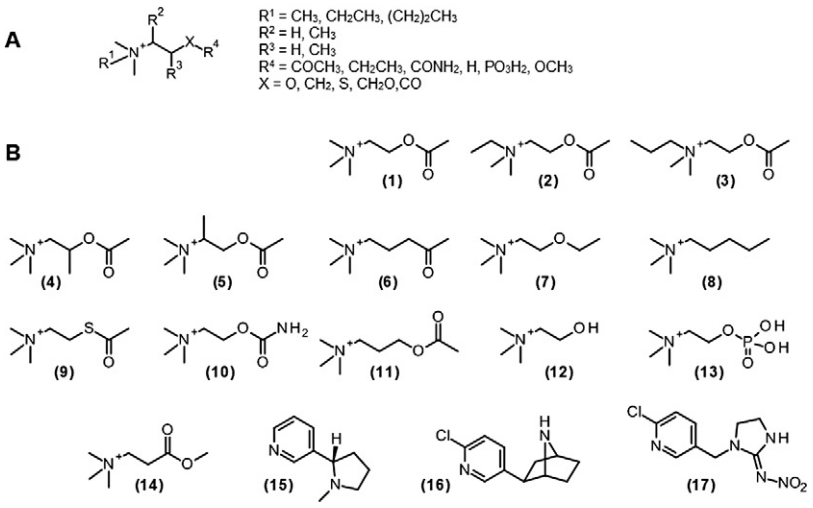

Figure 4. Chemical structures of acetylcholine (ACh) backbone derivatives for nicotinic acetylcholine (ACh) receptor (nAChR) structure-activity relationships and of nicotinoids and imidacloprid. (A) Overview of ACh modifications. (B) Chemical structures: (**1**) ACh; (**2**) acetyl-*N*-ethylcholine; (**3**) acetyl-*N*-propylcholine; (**4**) acetyl-β-methylcholine; (**5**) acetyl-α-methylcholine; (**6**) trimethyl-(4-oxopentyl) ammonium; (**7**) trimethyl-ethoxyethyl ammonium; (**8**) trimethyl-pentyl ammonium; (**9**) acetyl-thiocholine; (**10**) carbamoylcholine; (**11**) acetyl-homocholine; (**12**) choline; (**13**) phosphocholine; (**14**) trimethyl-(3-methoxy-3-oxopropyl) ammonium, (**15**) (–)-nicotine; (**16**) (1R,2R,4S)-(+)-6-(6-chloro-3-pyridyl)-7 azabicyclo[2.2.1] heptane, epibatidine; (**17**) imidacloprid.

Derivatives of R^4 and X: modification of the acetylcholine carboxylic ester. Replacement of the choline oxygen in acetylcholine (R^4) by a methylene group (**6**) led to a moderate increase in EC_{50} by a factor of 6 and 3 relative to acetylcholine for the *L. cuprina* α-subunit-containing Lcα1/Ggβ2 and Lcα2/Ggβ2, where this compound acted as a noncooperative partial and partially cooperative full agonist, respectively. In the case of Gga4/Ggb2, (**6**) behaved as a noncooperative full agonist, but the EC_{50} increased by a factor of 89 (Tables 1 and 2, Fig. 5A, D). Reduction of the acetylcholine carbonyl to a methylene group (**7**) resulted in a ligand of uniformly lower activity, with an increase in EC_{50} by 83-fold and 533-fold relative to acetylcholine for Lcα1/Ggβ2 and Ggα4/Ggβ2, and noncooperative partial agonist and negatively cooperative superagonist properties, respectively. Repeated application of (**7**) to Lcα2/Ggβ2 led to a marked receptor run-down of the ion channel function (data not shown). Therefore, EC_{50} determinations had to remain tentative and yielded a value of ~ 215 μM for this partial agonist, some 40-fold higher than for acetylcholine (Tables 1 and 2). A very similar picture was observed for the alkyl derivative (**8**), except that Lcα2/Ggβ2 experienced no receptor run-down and the EC_{50} values increased by factors of 25, 16 and 94 relative to acetylcholine, respectively. Furthermore, this derivative was a full agonist and not a superagonist for Ggα4/Ggβ2 (Tables 1 and 2). Replacement of the choline oxygen X by a sulphur atom [acetylthiocholine, (**9**)] as well as replacement of the acetyl group by a carbamoyl group [carbamoylcholine, (**10**)] led to a moderate increase in the EC_{50} values for Lcα1/Ggβ2 (6 and 18), Lcα2/Ggβ2 (17 and 30) and Ggα4/Ggβ2 (23 and 24) compared with the respective values for acetylcholine. These ligands showed no or only weak cooperativity in nAChR agonist action and behaved as partial (Lcα1/Ggβ2) and full agonists (Lcα2/Ggβ2, Ggα4/Ggβ2). For Lcα2/Ggβ2, repeated applications of acetylthiocholine led to pronounced irreversible receptor run-down (Fig. 6D), and both EC_{50} and I_{max} determination had to remain tentative, while Hill coefficients were not calculated (Tables 1 and 2).

Derivatives with modification of R^4 and X: mixed structural changes. Homologation of the ACh backbone from ethyl to n-propyl [acetylhomocholine, (**11**)] resulted in an agonist that showed a moderate loss in EC_{50} potency for Lcα1/Ggβ2 and Lcα2/Ggβ2 by a factor of 24 and 31 compared with ACh and acted as noncooperative and weakly cooperative partial agonist, respectively. For chicken Ggα4/Ggβ2, (**11**) was a

full agonist with negative cooperativity, but the rise in EC_{50} (factor 549) was much stronger than for the insect α-subunit-containing nAChRs. Removal of the ACh acetyl group yielding choline (**12**) led to a dramatic increase of EC_{50} compared with ACh for all three nAChRs of this study (Lcα1/Ggβ2, Lcα2/Ggβ2, Ggα4/Ggβ2), by factors of 506, 93 and 1296, respectively, and noncooperative partial agonist behaviour in the case of Lcα1/Ggβ2 and Ggα4/Ggβ2. For Lcα2/Ggβ2, receptor run-down was observed upon repeated application of choline (**12**) (Fig. 6B). Replacement of the acetyl group by a phosphate group led to a compound [phosphocholine, (**13**)] with agonist properties on all three receptors, albeit very weak on Lcα2/Ggβ2 (Fig. 3F). In the case of Lcα1/Ggβ2, phosphocholine (**13**) was a full agonist at very high concentrations (Fig. 3F). The detailed agonist properties of phosphocholine could not be assessed owing to low compound availability. The inversion of the ACh carboxylic group resulted in an agonist (**14**) that, remarkably, retained its potency and showed only low or moderate increases in EC_{50} relative to ACh for Lcα1/Ggβ2 and Lcα2/Ggβ2 (factors of 4 and 7, respectively), and which acted as a noncooperative partial agonist for Lcα1/Ggβ2 and a partially cooperative full agonist for Lcα2/Ggβ2. By contrast, the EC_{50} of (**14**) rose 405-fold compared with ACh for the chicken ganglionic nAChR Ggα4/Ggβ2, and acted as a negatively cooperative superagonist (Tables 1 and 2, Fig. 5A, F).

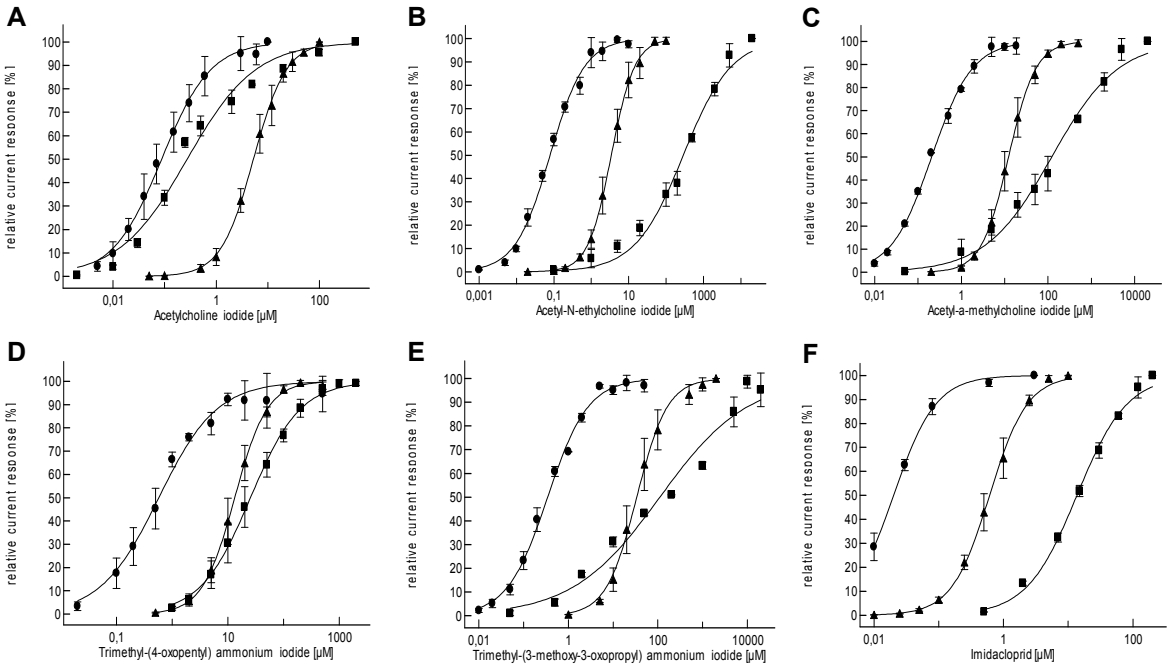

Figure 5: Hill curves of relative current responses of heteromeric nicotinic acetylcholine (ACh) receptors (nAChRs) with Lcα1 or Lcα2 or Ggα4 and Ggβ2 subunits: Lcα1/Ggβ2 (●), Lcα2/Ggβ2 (▲) and Ggα4/Ggβ2 (■). (A) Dose responses with ACh (**1**); (B) dose responses with acetyl-N-ethylcholine (**2**); (C) dose responses with acetyl-α-methylcholine (**5**); (D) dose responses with trimethyl-(4-oxopentyl) ammonium (**6**); (E) dose responses with trimethyl-(3-methoxy-3-oxypropyl)ammonium (**14**); (F) dose responses with imidacloprid (**17**). Error bars indicate the SD from 2-11 data points.

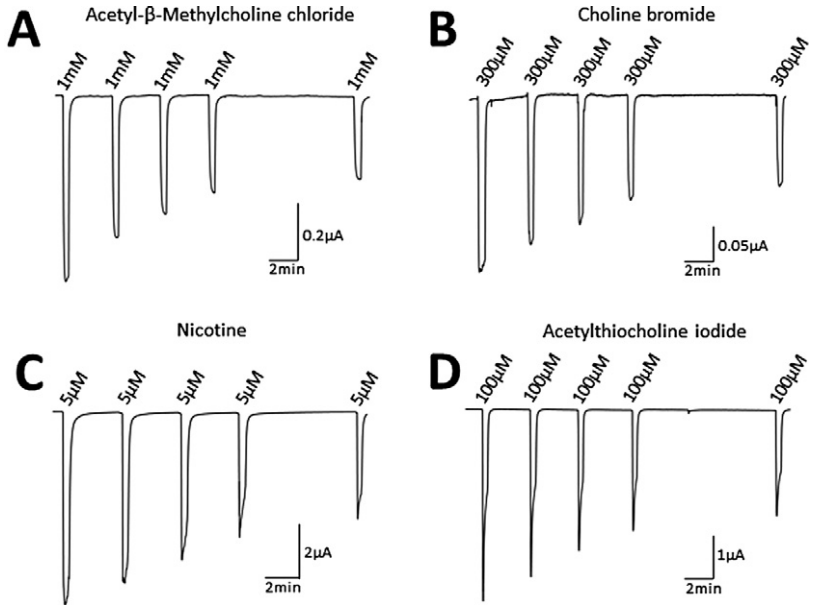

Figure 6: Repeated agonist application leading to receptor run-down of Lcα2/Ggβ2 expressed in *Xenopus* oocytes: (A) acetyl-β-methylcholine (**4**); (B) choline (**12**); (C) (-)-nicotine (**15**); (D) acetylthiocholine (**9**).

Table 1: *Xenopus* oocyte-expressed nicotinic acetylcholine receptors: EC_{50} values and Hill coefficients n_H of various agonists.

compound	EC_{50} Lcα1/Ggβ2 [μM]	n_H Lcα1/Ggβ2	EC_{50} Lcα2/Ggβ2 [μM]	n_H Lcα2/Ggβ2	EC_{50} Ggα4/Ggβ2 [μM]	n_H Ggα4/Ggβ2
Acetylcholine iodide (**1**)	0.08 +/- 0.03 (n=11)	1.05 +/- 0.18 (n=15)	5.37 +/- 1.15 (n=4)	1.46 +/- 0.19 (n=6)	0.29 +/- 0.08 (n=4)	0.69 +/- 0.06 (n=4)
Acetyl- N-ethylcholine iodide (**2**)	0.08 +/- 0.01 (n=4)	0.93 +/- 0.09 (n=4)	3.39 +/- 1.0 (n=4)	1.46 +/- 0.15 (n=4)	279.5 +/- 86 (n=3)	0.61 +/- 0.1 (n=4)
Acetyl-N-propylcholine iodide (**3**)	1.27+/- 0.23 (n=4)	0.80 +/- 0.07 (n=4)	54.1 +/- 5.1 (n=4)	1.41 +/- 0.1 (n=4)	29.2 +/- 5.5 (n=3)	0.46 +/- 0.03 (n=4)
Acetyl-β-methylcholine chloride (**4**)	22.9 +/- 6.0 (n=4)	1.24 +/- 0.18 (n=4)	approx[♯]. 455.0	n. g.	240.6 +/- 19.5 (n=4)	1.45 +/- 0.14 (n=4)
Acetyl-α-methylcholine iodide (**5**)	0.19 +/- 0.02 (n=5)	0.93 +/- 0.15 (n=5)	12.8 +/- 2.5 (n=3)	1.37 +/- 0.08 (n=4)	123.7 +/- 38.5 (n=4)	0.59 +/- 0.08 (n=7)
Trimethyl-(4-oxopentyl) ammonium iodide (**6**)	0.5 +/- 0.15 (n=4)	0.95 +/- 0.23 (n=4)	13.4 +/- 2.8 (n=4)	1.54 +/- 0.14 (n=4)	25.8 +/- 7.1 (n=3)	1.02 +/- 0.13 (n=4)
Trimethyl-ethoxyethyl ammonium bromide (**7**)	6.6 +/- 1.2 (n=4)	1.24 +/- 0.11 (n=4)	approx[♯]. 214.5	n. g.	154.7 +/- 20.6 (n=4)	0.67 +/- 0.08 (n=4)
Trimethyl-pentyl ammonium iodide (**8**)	2.0 +/- 0.84 (n=3)	0.88 +/- 0.07 (n=4)	86.1 +/- 4.4 (n=4)	1.57 +/- 0.14 (n=4)	27.3 +/- 0.1 (n=3)	0.79 +/- 0.06 (n=4)
Acetyl-thiocholine iodide (**9**)	0.44 +/- 0.1 (n=3)	0.96 +/- 0.05 (n=3)	approx[♯]. 89.7	n. g.	6.64 +/- 0.3 (n=3)	1.03 +/- 0.18 (n=3)
Carbamoylcholine chloride (**10**)	1.46 +/- 0.42 (n=4)	0.95 +/- 0.13 (n=4)	161.8 +/- 32.9 (n=3)	1.15 +/- 0.29 (n=5)	7.0 +/- 2.2 (n=4)	0.72 +/- 0.07 (n=5)
Acetyl-homocholine iodide(**11**)	1.88 +/- 0.61 (n=4)	0.92 +/- 0.15 (n=4)	165.2 +/- 38.7 (n=4)	1.35 +/- 0.1 (n=4)	159.3 +/- 44.1 (n=4)	0.70 +/- 0.03 (n=4)
Choline bromide (**12**)	40.5 +/- 6.5 (n=4)	1.12 +/- 0.18 (n=4)	approx[♯]. 502	n. g.	375.7 +/- 50.3 (n=5)	1.19 +/- 0.11 (n=5)
Trimethyl-(3-methoxy-3-oxopropyl) ammonium iodide (**14**)	0.34 +/- 0.04 (n=4)	0.98 +/- 0.02 (n=4)	36.0 +/- 12.0 (n=5)	1.32 +/- 0.17 (n=5)	117.6 +/- 23.1 (n=4)	0.44 +/- 0.02 (n=6)
Nicotine (**15**)	approx[♯]. 0.02	n. g.	approx[♯]. 0.94	n. g.	0.76[§]	1.1[§]
Epibatidine (**16**)	n. d.[*]	n. d.[*]	approx[♯]. 0.007	n. g.	n. d.[*§]	n. d.[*§]
Imidacloprid (**17**)	approx[♯]. 0.02	n. g.	0.62 +/- 0.12 (n=4)	1.52 +/- 0.1 (n=4)	13.8[§]	1.13[§]

The standard deviations and the number of repeats are indicated. [♯]: only approximate values, because of loss of signal ('receptor desensitization') upon repeated ligand application (see examples in Fig. 6); therefore, the corresponding Hill coefficients are also not given (n.g.). *: not determined (n. d.) due to irreversible agonist action. [§]: data taken from Dederer et al 2011. The compound numbers in parentheses refer to the structures in Fig. 4B.

Table 2: Ratio of compound EC_{50} values to EC_{50} of the natural ligand acetylcholine, and compound I_{max} values relative to I_{max} acetylcholine.

compound	$EC_{50}/EC_{50\ ACh}$ Lcα1/Ggβ2	I_{max} (%)* Lcα1/Ggβ2	$EC_{50}/EC_{50\ ACh}$ Lcα2/Ggβ2	I_{max} (%)* Lcα2/Ggβ2	$EC_{50}/EC_{50\ ACh}$ Ggα4/Ggβ2	I_{max} (%)* Ggα4/Ggβ2
Acetylcholine iodide (**1**)	1	100	1	100	1	100
Acetyl- N-ethylcholine iodide (**2**)	1	21.4[#] +/- 2.1 (n=4)	0.6	84.0[$] +/- 0.6 (n=4)	964	158.8[‡] +/- 26.0 (n=4)
Acetyl-N-propylcholine iodide (**3**)	15.9	35.0[#] +/- 7.0 (n=4)	10.1	63.0[$] +/- 8.4 (n=3)	100.7	56.4[‡] +/- 4.9 (n=4)
Acetyl-β-methylcholine chloride (**4**)	286.6	14.9[#] +/- 3.3 (n=4)	84.7[§]	approx. 1.7[$] +/- 0.5 (n=4)	829.6	87.9[‡] +/- 12.6 (n=4)
Acetyl-α-methylcholine iodide (**5**)	2.4	19.9[#] +/- 3.4 (n=5)	2.4	81.5[$] +/- 10.0 (n=4)	426.6	141.4[‡] +/- 12.0 (n=4)
Trimethyl-(4-oxopentyl) ammonium iodide (**6**)	6.25	48.5[#] +/- 2.5 (n=4)	2.5	103.0[$] +/- 1.6 (n=4)	89	113.9[‡] +/- 13.5 (n=4)
Trimethyl-ethoxyethyl ammonium bromide (**7**)	82.6	38.6[#] +/- 5.6 (n=4)	39.9[§]	approx. 40.5[$] +/- 6.9 (n=3)	533.4	120.4[‡] +/- 13.5 (n=4)
Trimethyl-pentyl ammonium iodide (**8**)	25.3	28.1[#] +/- 5.8 (n=4)	16	81.2[$] +/- 4.7 (n=3)	94.1	106.3[‡] +/- 7.1 (n=4)
Acetyl-thiocholine iodide (**9**)	5.5	57.6[#] +/- 4.3 (n=3)	16.7[§]	approx. 94.8[$] +/- 5.4 (n=4)	22.9	100.7[‡] +/- 3.9 (n=4)
Carbamoylcholine chloride (**10**)	18.3	78.9[#] +/- 16.3 (n=4)	30.1	97.3[$] +/- 12.7 (n=6)	24	112.6[‡] +/- 4.6 (n=4)
Acetyl-homocholine iodide(**11**)	23.5	17.9[#] +/- 4.0 (n=3)	30.8	51.0[$] +/- 6.9 (n=4)	549.4	107.4[‡] +/- 13.9 (n=4)
Choline bromide (**12**)	505.6	28.5[#] +/- 3.8 (n=4)	93.4[§]	approx. 9.9[$] +/- 1.7 (n=6)	1295.5	81.7[‡] +/- 3.3 (n=4)
Phosphocholine (**13**)	n. d.	> 90[#]	n. d.	< 5[$]	n. d.	> 40[‡]
Trimethyl-(3-methoxy-3-oxopropyl) ammonium iodide (**14**)	4.25	60.4[#] +/- 2.0 (n=4)	6.7	126.4[$] +/- 6.6 (n=4)	405.4	222.6[‡] +/- 18.4 (n=4)
Nicotine (**15**)	0.25[§]	approx. 11.6[#] +/- 2.8 (n=4)	0.18[§]	approx. 100.1[$] +/- 5.6 (n=4)	2.7	84.2[‡&]
Epibatidine (**16**)	n. d.	n.d.	0.001[§]	approx. 100.1[$] +/- 4.2 (n=4)	n. d.	n.d.
Imidacloprid (**17**)	0.25[§]	5.1[#] +/- 0.9 (n=4)	0.12	104.0[$] +/- 10.2 (n=4)	47.6	24.8[‡&]

*: relative to I_{max} acetylcholine set at 100%; I_{max} (%) was determined at the following saturating agonist concentrations: [#] (2) 5 µM, (3) 100 µM, (4) 250 µM, (5) 10 µM, (6) 20 µM, (7) 50 µM, (8) 100 µM, (9) 20 µM, (10) 50 µM, (11) 100 µM, (12) 500 µM, (13) 20 mM, (14) 20 µM, (15) 0.5 µM, (17) 3 µM. [$] (2) 100 µM, (3) 1 mM, (4) 1 mM, (5) 500 µM, (6) 200 µM, (7) 1 mM, (8) 1 mM, (9) 1 mM, (10) 10 mM, (11) 5 mM, (12) 5 mM, (13) 20 mM, (14) 5 mM, (15) 10 µM, (16) 0.5 µM, (17) 10 µM. [‡] (2) 20 mM, (3) 5 mM, (4) 5 mM, (5) 5 mM, (6) 1 mM, (7) 10 mM, (8) 2 mM, (9) 200 µM, (10) 5 mM, (11) 5 mM, (12) 10 mM, (13) 20 mM, (14) 20 mM, (15) 10 µM, (17) 200 µM. [§]: values based on the approximate EC_{50}s of table 1. n.d.: not determined. [&]: data taken from Dederer et al 2011. The standard deviations and the number of repeats are indicated. I_{max} up to 79.9%: partial agonist; 80%-119.9%: full agonist; ≥ 120%: superagonist. The compound numbers in parentheses refer to the structures in Fig. 4B.

Discussion

In the present report we describe the identification and full-length cloning of two nAChR subunits from the sheep blowfly *L. cuprina*. Protein sequence homology analyses suggest that they belong to the insect nAChR α1 and α2 subunit families, respectively. Trans-species hybrid receptors with chicken nAChR β2 subunits expressed in *Xenopus* oocytes resulted in ACh-induced currents up to the low and high μA range for Lcα1/Ggβ2 and Lcα2/Ggβ2 receptors, respectively. The EC_{50} values of 80 nM and 5.37 μM compared well with those determined for the cat flea and fruit fly nAChR α1 and α2 subunits in earlier studies (Bertrand et al., 1994; Dederer et al., 2011). The ACh sensitivity of Ggα4/Ggβ2 (Ballivet et al., 1988) determined in the present study was consistently in the nM range between the values for the *L. cuprina* α1 and α2 subunits (EC_{50} = 290 nM), and is in good agreement with earlier studies (Valera et al., 1992; Hussy et al., 1994), although somewhat higher values have also been reported (Shimomura et al., 2004; Dederer et al., 2011).

Further support for the notion of assigning these newly identified nAChR genes to the insect α1 and α2 groups comes from the α-bungarotoxin sensitivity of Lcα1 and the resistance of Lcα2, which are hallmarks of the respective gene families (Bertrand et al., 1994; Lansdell & Millar, 2000; Dederer et al., 2011). Epibatidine (**16**) irreversibly opens Lcα1/Ggβ2 ion channels at nM concentrations, while for Lcα2/Ggβ2 this alkaloid acts as an extremely potent (EC_{50} ~ 7 nM) desensitizing agonist. With respect to the responsiveness to the insecticidal market product imidacloprid (**17**), Lcα1/Ggβ2 possesses an EC_{50} in the nanomolar range, some 31-fold lower than that of Lcα2/Ggβ2, and some 690-fold lower than that of the chicken nAChR Ggα4/Ggβ2, which may be indicative for its role as neonicotinoid *in vivo* target (Dederer et al., 2011); however, while imidacloprid is a full agonist for Lcα2/Ggβ2, it is only a weak partial agonist for Lcα1/Ggβ2. The relationships of these findings for imidacloprid action *in vivo* remain to be determined.

In the past, structure-activity and structure–selectivity studies for agonist action on native and recombinant insect nAChRs have focused on neonicotinoid derivatives (Ihara et al., 2003; Tomizawa & Casida, 2003, 2005, 2009; Shimomura et al., 2004; Millar & Denholm, 2007; Thany et al., 2007, and references therein) and on

spinosyns (Sparks et al., 2001; Perry et al., 2007; Baxter et al., 2010; Kirst, 2010, and references therein). By contrast, systematic structure-activity relationships for the natural ligand ACh as a lead structure have not been reported for insect nAChRs. Surprisingly, in vertebrate nAChR research, studies on close ACh derivatives appear to be fragmentary (Dale, 1914; Ing et al., 1952; Clark et al., 1968; Shefter & Mautner, 1969; Aronstam & Buccafusco, 1982; Auerbach et al., 1983; Aronstam et al., 1988), particularly on defined recombinant receptors (Zhang et al., 1995; Jensen et al., 2003; Jonnala et al., 2003), while most of current nicotinic agonist research is focused on aromatic and/or alkaloid ligands derived from epibatidine, nicotine or cytosine and others (Breining, 2004; Bunnelle et al., 2004; Cassels et al., 2005; Gündisch & Eibl, 2011).

In the present study we have assembled a collection of 13 close ACh derivatives by chemical synthesis and compound purchase. The nAChR agonistic potential of this collection was analysed on the insect α subunit-containing Lcα1/Ggβ2 and Lcα2/Ggβ2, and in comparison, on the chicken neuronal receptor Ggα4/Ggβ2. The most remarkable differences with respect to the EC_{50} values were seen for acetyl-N-ethylcholine (**2**), which was as potent as or more potent on Lcα1/Ggβ2 and Lcα2/Ggβ2 than ACh, while the EC_{50} value of (**2**) on Ggα4/Ggβ2 rose by a factor of almost 1000 as compared to ACh. Similarly, the EC_{50}s of acetyl-α-methylcholine (**5**) for Lcα1/Ggβ2 and Lcα2/Ggβ2 rose only moderately (factor 2.4) relative to ACh, while in the case of Ggα4/Ggβ2 the increase in EC_{50} for (**5**) was about 427-fold as compared to ACh. A third derivative, where the difference in EC_{50} increases between insect and chicken receptors reached or exceeded the factor 100, was trimethyl-(3-methoxy-3-oxopropyl) ammonium (**14**), which basically corresponds to ACh with inverted ester function. Here, the EC_{50}s relative to ACh for Lcα1/Ggβ2 and Lcα2/Ggβ2 increased by modest 4.3 and 6.7-fold, while in the case of Ggα4/Ggβ2, this factor was 405.

Amongst the derivatives with less dramatic receptor potency differences, for acetylhomocholine (**11**) a dramatic increase of EC_{50} was noted for Ggα4/Ggβ2 (~550-fold) relative to ACh, while for the insect receptors Lcα1/Ggβ2 and Lcα2/Ggβ2, this was a more moderate 24-fold and 31-fold, respectively. Also trimethyl-(4-oxopentyl) ammonium (**6**) retained much of the ACh potency in Lcα1/Ggβ2 and Lcα2/Ggβ2 with EC_{50} increases of 6.3 and 2.4, respectively, relative to ACh, while

the chicken receptor potency dropped by a factor of 89. For all other derivatives differences of EC_{50} losses between the three receptor forms relative to ACh were generally near or below a factor of 10, which made these derivatives less instructive. In general, it appears that Ggα4/Ggβ2 is much less forgiving to the moderate structural changes on ACh that are displayed by the derivatives investigated in the present study. EC_{50} increases were generally 2–3 orders of magnitude, except for the very close derivatives acetylthiocholine (**9**) and carbamoylcholine (**10**) (Tables 1 and 2). By contrast, the less dramatic or non-existent EC_{50} rises for the insect receptors Lcα1/Ggβ2 and Lcα2/Ggβ2 suggest that particularly substitution at the ACh quarternary ammonium, branching and the length of the ACh alkyl chain between ammonium and ester function, ketone analogues of ACh as well as the inverted ACh ester structure could be sources for selectivity between insect and chicken nAChRs investigated in the present study and therefore logical entry points for derivatization programmes. As fruit fly and cat flea nicotinic receptor hybrid receptor data for the insect α1 subunit and neonicotinoids are in better accord with the insect *in vivo* toxicity of these compounds than for the insect α2 subunit (Dederer et al., 2011), and as neonicotinoid target site resistance has been shown to be associated with α1 subunit mutations in *Drosophila* (Perry et al., 2008) and in *Nilaparvata* (Liu et al., 2005, 2006, 2009), such derivatization programmes should initially focus on the insectα1/chickenβ2 nicotinic receptor combinations.

The last decade has seen considerable progress in the structural biology of nAChR ligand-binding domains, in part with co-crystallized natural and drug ligands (Brejc et al., 2001; Celie et al., 2004; Rucktooa et al., 2009; Li et al., 2011). These developments also paved the way for molecular modelling of receptors without structural information as well as *in silico* pharmacophor and docking studies, with the aim of achieving structure-guided design of nAChR-addressing drugs (Taylor et al., 2007; Ulens et al., 2009; Abin-Carriquiry et al., 2010; Akdemir et al., 2011; Kombo et al., 2012). The structure-activity and structure-selectivity relationships of ACh derivatives uncovered in the present study are ideal entry points for corresponding *in silico* studies on insect nAChR subunit-containing structural models (Tomizawa et al., 2011).

In summary, in addition to the identification and characterization of the previously unknown blowfly nAChR α subunits, our study has revealed hitherto

unknown structure-activity and structure-selectivity relationships of a comprehensive set of ACh derivatives on *L. cuprina* α1, *L. cuprina* α2 and chicken α4-containing nAChR hybrid receptors with chicken β2. Future studies will address other vertebrate nAChRs and the more extended structural space of ACh to probe in depth for insect nAChR selectivity potential. Both the most selective derivatives and the non-selective compounds arising of such an approach may serve for guidance in the quest for selective insecticides, either by wet bench or *in silico* methods.

Conflict of interest

The authors declare the following competing financial interest: all authors are employed by MSD Animal Health Innovation GmbH.

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Ich erkläre hiermit, dass ich, Dipl.-Biochemikerin Helene Dederer, die zur Promotion eingereichte Arbeit selbständig verfasst, nur die angegebenen Quellen und Hilfsmittel benutzt und wörtlich oder inhaltlich übernommene Stellen als solche gekennzeichnet habe.

Bingen, den 24.04.2013

Helene Dederer