α-Conotoxins active at α3-containing nicotinic acetylcholine receptors and their molecular determinants for selective inhibition

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Abstract
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α-Conotoxins active at α3-containing nicotinic acetylcholine receptors and their molecular determinants for selective inhibition

Running title: Conotoxins targeting α3-containing nAChRs

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Abbreviations: AChBP, acetylcholine binding protein; CNS, central nervous system; DRG, dorsal root ganglion; MD, molecular dynamics; nAChRs, nicotinic acetylcholine receptors; PNS, peripheral nervous system
Abstract
Neuronal α3-containing nicotinic acetylcholine receptors (nAChRs) in the peripheral nervous system (PNS) and non-neuronal tissues are implicated in a number of severe disease conditions ranging from cancer to cardiovascular diseases, and chronic pain. However, despite the physiological characterization of mouse models and cell lines, the precise pathophysiology of nAChRs outside the central nervous system (CNS) remains not well understood, in part because there is a lack of subtype-selective antagonists. α-Conotoxins isolated from cone snail venom exhibit characteristic individual selectivity profiles for nAChRs and, therefore, are excellent tools to study the determinants for nAChR-antagonist interactions. Given that human α3β4 subtype-selective α-conotoxins are scarce and this is a major nAChR subtype in the PNS, the design of new peptides targeting this nAChR subtype is desirable. Recent studies using α-conotoxins RegIIA and AuIB, in combination with nAChR site-directed mutagenesis and computational modeling have shed light onto specific nAChR residues which determine selectivity for the human α3β2 and α3β4 subtypes. Publications describing the selectivity profiles and binding site of other α-conotoxins confirm that subtype-selective nAChR antagonists often work through common mechanisms by interacting with the same structural components and sites on the receptor.

Key Words
Conotoxins; nicotinic acetylcholine receptors; receptor antagonists; binding selectivity; electrophysiology; site-directed mutagenesis; homology modeling; molecular dynamics; peripheral nervous system
**Structure and physiology of neuronal nAChRs**

The cation selective nAChRs are pentameric ligand-gated ion channels of the Cys-loop receptor superfamily (Fig. 1), which also includes γ-aminobutyric acid type A (GABA\(\alpha\))-; glycine- and serotonin (5-HT\(3\)) receptors (Changeux, 2012). Channel opening is triggered by binding of the endogenous ligand acetylcholine (ACh) or exogenous ligands such as nicotine to the binding site at the interface of two adjacent subunits (Albuquerque et al., 2009; Cecchini et al., 2015; Itier et al., 2001; Jensen et al., 2005).

Seventeen nAChR subunits have been identified in vertebrate species (\(\alpha_1\)–\(\alpha_{10}\), \(\beta_1\)–\(\beta_4\), \(\gamma\), \(\delta\) and \(\varepsilon\)). All of these subunits, except \(\alpha_8\), which has been identified only in avian species, are expressed in humans and in other mammalian species (Lukas et al., 1999; McGehee et al., 1995; Millar et al., 2009). Nine \(\alpha\) (2-10) and three \(\beta\) (2-4) subunits are generally classified as neuronal nAChRs (Albuquerque et al., 2009; Millar et al., 2009). \(\alpha_7\) and \(\alpha_9\) nAChR subunits form functional homopentameric receptors, but most native nAChR subtypes consist of different subunits assembling to heteropentameric receptors (Fig. 1A, C).

Neuronal nAChRs are present in a variety of CNS neurons, and also in autonomic ganglion neurons, vagal afferent neurons, and adrenal chromaffin cells of the PNS (Campanucci et al., 2010; Cooper, 2001; Rudchenko et al., 2014; Sargent, 1993). Most neuronal nAChRs are localized presynaptically where they modulate neurotransmitter release either through the depolarization of the presynaptic bouton (Dani et al., 2007) and/or an increase of intracellular Ca\(^{2+}\) concentration (Albuquerque et al., 2009). Neuronal nAChR subtypes are also located postsynaptically, where nAChR activation initiates depolarization and action potential firing (Dani et al., 2007).

Numerous nAChR subunit configurations are expressed in the CNS (Gotti et al., 2007; Zoli et al., 2015) where they have been implicated in neurological disorders such as Parkinson’s and Alzheimer’s disease, schizophrenia, Tourette’s syndrome and epilepsy, as well as opiate and nicotine addiction (Changeux, 2010; Dani et al., 2007; Hurst et al., 2013; Lindstrom, 1997; Muldoon et al., 2014; Ripoll et al., 2004). Furthermore, a variety of nAChR subunits are expressed in the PNS and non-neuronal cells, leading to a vast array of subunit combinations. This diversity results in the customized pharmacology and distinct function of non-CNS nAChRs, including \(\alpha_3\)-containing nAChRs. The focus of this review is on \(\alpha_3\) subunit-containing
nAChRs, as their physiological functions are not well understood due to the lack of subtype-selective antagonists.

**α3β4 and α3β2 nAChR subtypes expressed in the PNS and non-neuronal cells**

In the PNS, the α3 subunit is widely expressed in autonomic ganglia (Jensen et al., 2005; Millar et al., 2009; Zoli et al., 2015), adrenal chromaffin cells and enteric nervous system (Tachikawa et al., 2001; Zhou et al., 2002), preferentially forming heteromeric nAChRs with the β4 subunit (Table 1). Studies using α3 knockout mice indicate that this subunit is an essential component of the nAChRs for mediating normal function of the autonomic nervous system. Phenotypic abnormalities of the mice include impaired growth and increased mortality after weaning, dysfunctionsally enlarged bladder, urinary stones and widely dilated ocular pupils (Xu et al., 1999a). Detailed histological examination of the α3-null mice revealed no significant abnormalities in the brain or peripheral tissues, whereas inflammation was prominently observed in the urinary bladder. Electrophysiological recordings showed reduced open probability of ACh-activated single channel currents in neurons of the superior cervical ganglia (Xu et al., 1999a).

Furthermore, postganglionic sympathetic neurons of α3-null mice are devoid of excitatory synaptic potentials, confirming postsynaptic α3-containing receptors are a crucial component for synaptic transmission in autonomic ganglia (Rassadi et al., 2005). Interestingly, a β2-β4 double knockout mouse showed phenotypic abnormalities and physiological impairments similar to the α3-null mouse, despite both β2 and β4 single knockout mice appearing superficially normal (Xu et al., 1999b). This finding suggests an apparent redundancy between β2 and β4 in forming physiologically intact receptors. Indeed, no overt physiological abnormalities were identified in β2-null mice (Wang et al., 2005). In contrast, ion channel function in the superior cervical ganglion and bladder contractility were functionally impaired in the β4-null mouse (Xu et al., 1999b), indicating that α3 and β4 subunits are the major components of ganglionic nAChRs.

ACh release in the adrenal medulla, triggered by stimulation of the splanchnic nerve from the sympathetic nervous system, activates nAChRs in chromaffin cells. Opening of the nAChRs causes membrane depolarization, triggering catecholamine secretion (Sala et al., 2008).

Expression analysis by in situ hybridization indicated that all chromaffin cells co-express α3, α5 and β4 transcripts (Campos-Caro et al., 1997), and as these subunits can form functional
nAChRs, it is postulated they are responsible for the release of catecholamines (Sala et al., 2008).

The \( \alpha_3 \beta_4 \) nAChR has been identified as the major subtype expressed in both sympathetic and parasympathetic major pelvic ganglion neurons of the male rat, as ACh-induced currents were inhibited by the \( \alpha_3 \beta_4 \) nAChR antagonists, mecamylamine and \( \alpha \)-conotoxin AuIB (Park et al., 2006). Rat superior cervical ganglia of the sympathetic nervous system also reportedly express \( \alpha_3 \)-containing receptors, which are associated with \( \beta_2 \) and/or \( \beta_4 \) subunits (Del Signore et al., 2004).

Multiple distinct subtypes of nAChRs are expressed and pharmacologically active in cultured dorsal root ganglion (DRG) neurons (Dube et al., 2005; Genzen et al., 2001; Hone et al., 2012) (Table 1). Rodent DRG neurons can be divided into four different subclasses based on their nAChR expression, with one of them predominantly expressing \( \beta_4 \)-containing nAChRs with \( \alpha_3 \) and \( \alpha_6 \) (Smith et al., 2013). Given that \( \alpha_3 \)-containing nAChRs, including the \( \alpha_3 \beta_4 \) subtype, are predominantly expressed in nociceptive DRG neurons (Fucile et al., 2005; Spies et al., 2006), it suggests this subtype may be involved in neuronal signaling of nociception and inflammation. A recent electrophysiological study on cutaneous rat DRG neurons showing that inflammation induces an increase in current density of slow nicotine-evoked currents is consistent with the involvement of \( \alpha_3/\beta_4 \)-containing nAChRs (Zhang et al., 2015). Furthermore, in cultured mouse sympathetic neurons and adrenal chromaffin cells, \( \alpha_3 \)-containing nAChRs were identified as key components for the hyperglycemia-induced inactivation of nAChR-mediated currents. This finding is relevant in the context of diabetes, as impaired sympathetic neuronal signaling of the autonomic nervous system is commonly observed among people with diabetes (Campanucci et al., 2010; Rudchenko et al., 2014).

In addition to their presence in many cells of the PNS, \( \alpha_3 \beta_4 \) and \( \alpha_3 \beta_2 \) nAChRs have also been detected in a large variety of non-neuronal cells such as human vascular endothelial cells, epithelial cells, lymphocytes, macrophages, mast cells, granulocytes, keratinocytes, fibroblasts, microglia and astrocytes (Arias et al., 2009; Gotti et al., 2004; Graham et al., 2003; Sharma et al., 2002; Wessler et al., 2008) (Table 1). For example, \( \alpha_3 \)-containing nAChRs play a pivotal role in regulating the inflammatory responses in endothelial cells and macrophages (Yang et al., 2016). It can be expected that these subtypes are expressed in other tissues, yet to be analyzed (Egleton et al., 2008; Sharma et al., 2002).
To add to the complexity of non-neuronal distribution of nAChRs, their expression pattern not only varies according to phenotypic cell function but also changes in response to internal and external environmental conditions. For example, it has been shown that during the maturation of keratinocytes, steroid hormones trigger changes in the expression pattern of nicotinic and muscarinic AChRs, resulting in the formation of completely different subtypes (Wessler et al., 2008).

Given that Ca\(^{2+}\) appears to be a primary mediator of nAChR signaling in non-neuronal cells, the downstream effects are likely to be diverse as well and dependent on the cell type. Activation of \(\alpha_3\)-containing nAChRs in these cells can control a number of general cellular processes like cell migration, survival, differentiation, proliferation, regulation of gene expression and other cell type-specific roles in an autocrine and paracrine manner (Egleton et al., 2008; Sharma et al., 2002; Wessler et al., 2008). Not surprisingly, non-neuronal signaling of these nAChRs has considerable implications for pathophysiological conditions such as cancer and cardiovascular disease. Due to their involvement in cell proliferation, non-neuronal nAChRs are linked to nicotine-induced cancer. Although nicotine is believed to stimulate cell proliferation via signaling pathways mainly involving the \(\alpha_7\) subtype, the anti-apoptotic activity of nicotine in bronchial epithelial cells is mediated by \(\alpha_3\)- and \(\alpha_4\)-containing nAChRs (Egleton et al., 2008; West et al., 2003). Furthermore, epidemiologic data suggests the role of \(\alpha_3\)-containing nAChRs in tobacco-related cancers in smokers (Hallden et al., 2016).

From physiology to pharmacology
Molecular techniques such as in situ hybridization, immunoassays as well as studies on transgenic mice contributed significant insight into the expression and distribution of nAChR subunits and their physiological role. However, these studies fail to provide detailed knowledge about the tissue-specific physiological role of individual nAChR subtypes. To this end, pharmacological studies can contribute crucial information in filling this gap. For example, at presynaptic terminals the \(\alpha_3\beta_4\) nAChR subtype has been shown to induce noradrenaline release, whereas \(\beta_2\)-containing receptors with \(\alpha_3\) and \(\alpha_6\) subunits induce dopamine release (Kulak et al., 2001; Soliakov et al., 1996). Subtype selective \(\alpha\) -conotoxins such as AuIB and MII (described in more detail below) can provide direct evidence of the physiological functions of nAChRs such as the inhibition of noradrenaline and dopamine release, respectively (Luo et al., 1998), or the
specific role of α3β4 in small cell lung carcinomas (Improgo et al., 2013). Furthermore, some α-conotoxins that specifically target nAChR subtypes have been shown to be effective at reducing mechanical allodynia in neuropathic pain models. In vitro patch clamp electrophysiology of primary afferent synaptic transmission pharmacologically showed that the mode of action of these conotoxins presumably involves the inhibition of nAChRs containing α3 and α7 subunits (Napier et al., 2012). These findings provided valuable information about receptor targets for the effective treatment of neuropathic pain.

Conotoxins from cone snail venom, a rich source of pharmacological compounds – a short overview

Conopeptides are peptides isolated from the venom of predatory marine snails of the genus Conus, commonly called cone snails. More than 800 species of cone snails are known (Puillandre et al., 2015), and the venom of each species is a complex and unique mixture of at least 200 distinct peptides (Lavergne et al., 2015; Olivera, 2006). Cone snails inject their venom via a hollow harpoon-like tooth to immobilize their prey; mostly worms, mollusks and other invertebrates. Approximately 50 species of cone snail prey on fish and the venom of one species (C. geographus) is even dangerous to humans (Gray et al., 1988; Kohn, 2016; Livett et al., 2004) with a human lethal dose estimated to be between 0.0038 and 0.029 mg/kg (Dutertre et al., 2014). Of the estimated >100,000 peptides present in Conus venoms, to date only a relatively small portion (< 0.1 %) have been structurally and pharmacologically characterized (Akondi et al., 2014; Lewis et al., 2012; Schroeder et al., 2012). Thus, cone snail venoms are still a vast source of potential pharmacologically active substances. The short disulfide-rich conotoxins, which specifically target different voltage- and ligand-gated ion channels, are the best characterized biologically active cone snail venom peptides. For an in-depth overview of the different classes of conotoxins and their pharmacological profiles, see the following comprehensive reviews (Akondi et al., 2014; Becker et al., 2008; Lewis et al., 2012; Robinson et al., 2014).

α-Conotoxins, a class of conotoxins active at nAChRs

Peptides with pharmacological activity at nAChRs have been identified in several conotoxin superfamilies (Akondi et al., 2014), but the largest and best known subgroup of conotoxins
inhibiting nAChRs are the α-conotoxins. α-Conotoxins are 12-20 amino acid disulfide-bonded peptides and represent the largest known group of nAChR antagonists. Some α-conotoxins exhibit high selectivity towards specific nAChR subtypes. Consequently, they are excellent tools to pharmacologically study the distribution and functional role of nAChR subtypes in specific tissues and the nervous system.

The structure of α-conotoxins is defined by the two disulfide bonds, contributed by four cysteine residues (C\textsubscript{I-IV}) (Fig. 2). The characteristic framework for α-conotoxins is C\textsubscript{I}C\textsubscript{II}X\textsubscript{m}C\textsubscript{III}X\textsubscript{n}C\textsubscript{IV}, where X\textsubscript{m} and X\textsubscript{n} refer to the number of non-cysteine residues which define loops 1 and 2 of the peptide. Loop 1 comprises of four residues (m = 4) and loop 2 has between three and seven residues (n = 3, 6 or 7) (Millard et al., 2004). Due to the shuffling of disulfide bonds, α-conotoxins can fold into three different isomers with completely altered three-dimensional structure. Most native and pharmacologically active α-conotoxins adopt the globular isomer with a C\textsubscript{I}-C\textsubscript{III} and C\textsubscript{II}-C\textsubscript{IV} connectivity (Fig. 2). The two other possible isomers are the ribbon (C\textsubscript{I}-C\textsubscript{IV} and C\textsubscript{II}-C\textsubscript{III}) and bead (C\textsubscript{I}-C\textsubscript{II} and C\textsubscript{III}-C\textsubscript{IV}) (Millard et al., 2004; Muttenthaler et al., 2011). Comprehensive reviews of α-conotoxins and their pharmacological activities at muscle and neuronal nAChRs subtypes have been published (Azam et al., 2009; Lebbe et al., 2014; Lin et al., 2016a; Muttenthaler et al., 2011).

**Pharmacologically relevant α-conotoxins active at α3β2 nAChRs**

The only known α-conotoxin with a 4/3 disulfide framework active at α3β2 nAChR, is α-conotoxin ImI, isolated from the venom of the worm hunting cone snail *C. imperialis*. It was reported to be a selective antagonist for neuronal over muscle nAChRs at the mouse neuromuscular junction (McIntosh et al., 1994). It most potently blocked the human α3β2 subtype with an IC\textsubscript{50} of 40.8 nM while also being active at human α7 (IC\textsubscript{50} 595 nM), and weakly active at α3β4 (IC\textsubscript{50} 3.39 μM) (Ellison et al., 2004) (Table 2). BuIA, from the venom of the fish-eating cone snail *C. bullatus*, with an uncommon 4/4 spacing of disulfide loops, potently inhibits several rat nAChR subtypes, including α3β2 with an IC\textsubscript{50} of 5.7 nM (Azam et al., 2005) (Table 2).

A large number of α-conotoxins acting on α3β2 are from the 4/7 cysteine framework group. One of the earliest and well-characterized 4/7 α-conotoxins, MII from *C. magus*, was shown to have a remarkable selectivity for α3β2 with an IC\textsubscript{50} in the low nanomolar range,
whereas other nAChR subtypes are >200-fold less sensitive (Cartier et al., 1996; Dowell et al., 2003; Harvey et al., 1997). α-Conotoxin GIC isolated from the venom of C. geographus selectively and potently inhibits human α3β2 with an IC50 of 1.1 nM, whereas potency at human α3β4 is 755 nM (Table 2). Furthermore, it has a remarkable 100,000-fold selectivity for α3β2 over muscle nAChRs (Lin et al., 2016b; McIntosh et al., 2002). Another 4/7 α-conotoxin with nanomolar affinity for α3β2 nAChRs is PIA from C. purpurascens. Interestingly, PIA displayed a 75-fold higher affinity for rat α6-containing nAChRs (specifically the α6/α3β2β3 subtype) than for rat α3β2 (Dowell et al., 2003).

Luo et al. recently reported the discovery of 4/7 α-conotoxin LvIA as a potent α3β2-selective conotoxin which exhibited low nanomolar potency at rat α3β2 (IC50 8.7 nM) whereas its IC50 values were >100 nM at α6/α3β2β3, α6/α3β4 and α3β4 nAChRs (Luo et al., 2014). At the homologous human nAChR subtypes, an even clearer preference for α3β2 over α6/α3β2β3 (IC50 17.5 nM compared to 5342 nM) was observed.

**α-Conotoxins pharmacologically active at α3β4 nAChRs and their molecular determinants for selectivity**

To date, only few pharmacological compounds have been discovered that selectively block the α3β4 nAChR subtype. As this is the predominant subtype in the peripheral nervous system and its involvement in several important pathophysiological processes has been shown, there remains a need for pharmacological compounds with high affinity and selectivity for this subtype.

The first α3β4-selective α-conotoxin described was the 4/6 toxin AuIB from C. aulicus (Luo et al., 1998) (Fig. 2B). AuIB inhibits rat α3β4 with >100-fold higher potency than other nAChR subtypes, even though the potency at α3β4 itself is relatively low (IC50 = 0.75 μM) (Table 2). Closer analysis of α3β4-mediated ACh-evoked current inhibition by AuIB revealed properties unique to this peptide. First, the non-native ribbon isomer of AuIB exhibits a lower IC50 at inhibiting α3β4 compared to the native globular isomer. This is in contrast to the general assumption that conotoxins lose activity when not folded in the native form. However, analysis of concentration-dependent inhibition of ACh-evoked currents mediated by α3β4 revealed the maximal block (efficacy) of the ribbon isomer is only ~50%, whereas complete block was achieved by sufficiently high concentrations of the globular isomer (Grishin et al., 2010). Furthermore, ribbon AuIB behaves as a competitive antagonist and its inhibition is dependent on
the receptor subunit stoichiometry. In contrast, inhibition by the globular isomer is independent of receptor stoichiometry, and surprisingly, globular AuIB inhibits α3β4 by a non-competitive antagonism, which is unusual for α-conotoxins. Molecular docking simulation suggested that ribbon AuIB binds to the ACh-binding pocket of the α3(3)β4(2) nAChR stoichiometry (Grishin et al., 2010), whereas globular AuIB is likely to bind to both α3(3)β4(2) and α3(2)β4(3) stoichiometries at a location outside the ACh-binding pocket.

In a subsequent study, the mechanism and structural determinants of AuIB binding to α3β4 were analyzed in more detail (Grishin et al., 2013). Alanine scan mutagenesis revealed two AuIB analogues, AuIB[P6A] and AuIB[F9A], did not inhibit α3β4 nAChR. The AuIB[P6A] analogue lost its secondary structure and consequently the ability to inhibit α3β4, but the AuIB[F9A] analogue retained its globular structure and the loss in activity was due to disruption of the specific peptide-receptor pairwise contacts. Molecular docking and receptor mutagenesis revealed that the main determinants of AuIB activity at α3β4 lie at the β4 subunit. Residues W59 and K61 form a binding pocket for F9 of AuIB in which its aromatic ring is inserted between the two aforementioned β4 residues and stabilized by them. Alanine substitution of K61 reduced inhibition, whereas substitution of W59 completely abolished inhibition, suggesting W59 is the main determinant of peptide activity. Interestingly, W59 is common to all neuronal nAChRs and hence cannot determine AuIB selectivity by itself. K61, however, is unique to the β4 subunit, and likely determines AuIB selectivity for α3β4 (Grishin et al., 2013).

Luo et al. reported the discovery of conotoxin TxID, the most potent α-conotoxin for α3β4 nAChRs described to date (Luo et al., 2013). TxID inhibited rat α3β4 nAChRs with an IC₅₀ of 12.5 nM and the closely related α6/α3β4 nAChRs with 7-fold less potency (IC₅₀ 94 nM), but exhibited minimal activity at other nAChR subtypes. Interestingly, TxID is the only α-conotoxin sharing the 4/6 disulfide framework with AuIB, but is 60-fold more potent than AuIB at α3β4 nAChRs.

Other α-conotoxins inhibiting ACh-evoked currents mediated by α3β4 with low nanomolar IC₅₀ have been reported but none are selective for this nAChR subtype. Therefore, effort has been focused on understanding the selectivity profile of α-conotoxins and, more specifically, the molecular determinants for specificity towards α3β4. An example is the 4/7 α-conotoxin RegIIA, isolated from C. regius (Fig. 2C). RegIIA exhibited low IC₅₀ values at rat α3β4 (97 nM) and α3β2 (33 nM), and human α7 (103 nM) nAChRs (Franco et al., 2012). Therefore, despite the
potent activity at α3β4, RegIIA is far from being a selective pharmacological probe. Alanine-scanning mutagenesis revealed [N11A] and [N12A] analogues of RegIIA were 3-fold more selective for α3β4 compared to α3β2, and the double-alanine RegIIA[N11A,N12A] analogue was even more selective for α3β4 with an IC50 of 370 nM (Kompella et al., 2015b). Although less potent at α3β4 compared to TxID, RegIIA[N11A,N12A] exhibits a >14-fold selectivity for α3β4 over the α6/α3β4 subtype (IC50 ~5 µM) (Kompella et al., 2015b).

Species-specific activity differences of α-conotoxins help elucidate key residues for selective binding to α3-containing nAChR subtypes

An impediment for the effective development of nAChR subtype-selective α-conotoxins as pharmacological probes with therapeutic potential is the fact that most α-conotoxins have only been characterized in heterologous expression systems using cloned nAChR subunits of one species, usually rat. However, there is growing evidence that α-conotoxins can exhibit considerably different potencies on homologous nAChR subtypes of different species (Azam et al., 2012; Azam et al., 2015; Yu et al., 2013).

Interestingly, α-conotoxin RegIIA was significantly less potent at the human α3β2 nAChR subtype compared to rat, whereas at α3β4, no species-specific differences in sensitivity were observed (Kompella et al., 2015a). As the homologous receptor subtypes of rat and human are overall relatively conserved, it is believed the binding site of RegIIA at α3β2 and α3β4 nAChRs overlaps with the agonist binding site (Kompella et al., 2015a). Therefore, only few residues in the ACh binding domains that differ between the species were predicted to play a role in determining selectivity. It was predicted that the determinants for the species difference would reside on the β subunit, because at α3β2 no species difference in activity was observed. The extracellular domains of the β4 subunit differ in only three residues, and surprisingly, when any of these residues in the rat β4 subunit was mutated to the human counterpart only a modest loss in activity was observed. Conversely, exchange of a single residue in loop F of the α3 subunit (Q198 to P) was sufficient to reduce the affinity of RegIIA to a value similar to that observed for the human nAChR. Mutagenesis experiments and MD simulations showed that the residue exchange causes structural changes at the ACh binding site by reducing the flexibility of nearby receptor residues and preventing them from effective interaction with RegIIA residues due to steric hindrance (Kompella et al., 2015a). A similar effect has been reported for α4β2, whereby
the reverse mutation at the homologous position in rat α4, P195Q, enhanced α-conotoxin TxA potency at α4β2 by increasing the number of contacts between toxin and receptor (Beissner et al., 2012).

As mentioned above, AuIB was the first rat α3β4 selective α-conotoxin discovered and it has represented a prime example for a specific antagonist of this receptor subtype. However, AuIB was largely inactive with no activity observed at 1 µM when tested on human α3β4 (compared to an IC50 of 0.75 µM at rat α3β4) and at 30 µM only ~20% of ACh-evoked current amplitude mediated by human α3β4 was inhibited, indicating that the IC50 is > 40-fold higher than for rat α3β4. At the human α3β2 subtype, AuIB exhibited a similar low inhibitory potency (Cuny et al., 2016).

**Molecular determinants in the agonist binding loops of human α3β2 and α3β4 nAChRs that define subtype selectivity of α-conotoxins**

As the extracellular domains of human β2 and β4 subunits are reasonably conserved, mutational studies on non-conserved amino acids have identified critical residues for the differences in nAChR subtype response to α-conotoxins. With molecular modeling and molecular dynamic (MD) simulations, the lack of activity of AuIB at human α3β4 nAChR could be interpreted on a molecular level. At the rat α3β4 nAChR, residue F9 of AuIB closely interacts with residues W59 and K61 of the β4 subunit WLK (Trp-Leu-Lys) pocket, hypothetically via π-π and cation-π interactions. In contrast, at the human α3β4 nAChR residue F9 of AuIB only binds outside the WLK pocket (Fig. 3A), where although interaction is still possible with β4 residues K59 and L119, the crucial π-π interaction with W57 is absent (Cuny et al., 2016). At the rat homologue, the β4 W59 residue was indispensable for AuIB inhibition and K61 appeared to play an auxiliary role (Grishin et al., 2013). Hence, the lack of interaction between AuIB F9 and human β4 W57 could explain the loss of AuIB activity at the human α3β4 subtype.

At human α3β2, molecular modeling revealed contacts between AuIB and β2 W57, T59 and other residues. Although AuIB F9 can form the crucial π-π interaction with W57 in loop D, unlike β4 K59 the hydrophilic side chain of β2 residue T59 does not form an effective binding pocket for AuIB F9 because its interaction with the aromatic phenyl ring of AuIB F9 is energetically unfavorable (Fig. 3B) (Cuny et al., 2016). In summary, AuIB at hα3β2 and hα3β4
nAChRs is stabilized ineffectively due to the lack of key interactions between AuIB F9 and β subunit agonist binding loop residues.

The key residues for receptor binding, which consequently dictate the selectivity profile, are not precisely the same for all 4/7 α-conotoxins despite overall structural similarity among these peptides. For α-conotoxins MII, \textit{PnIA} and GID, which all exhibit high potency at α3β2, residues β2-F119 and β2-V111 have a major impact on peptide binding. When the bulky side chains of those residues in β2 were exchanged to Ala or Gly, the peptides exhibited increased inhibitory activity for the mutant α3β2 nAChRs, indicating their affinity to the receptor was enhanced. Investigation of \textit{PnIA} analogues indicated that high affinity binding to aforementioned mutated β2 residues was conferred by amino acids at position 10 with long side chains (Dutertre \textit{et al.}, 2005).

For α-conotoxin RegIIA, the human β2 and β4 subunit selectivity was dependent on two key residues located at position 59 in loop D and position 113 in loop E, respectively. Exchange of β2 T59 to K, increased RegIIA potency at the mutant human α3β2 similar to the potency at α3β4, whereas the opposite exchange of β4 K59 to T resulted in ~60-fold decrease in potency compared to wild-type human α3β4 (Cuny \textit{et al.}, 2016). Swapping loop E residues S113 of β2 subunit with R113 of β4 subunit considerably improved the sensitivity of the receptor to RegIIA (twice as sensitive as wild-type α3β4). Conversely, β4 R113S mutation reduced the sensitivity to RegIIA by ~20-fold compared to wild-type human α3β4 (Cuny \textit{et al.}, 2016). To our knowledge, this study was the first in in identifying β4 R113 as a key determinant for antagonist binding at human nAChRs.

The sensitivity changes of the mutant receptors compared to their wild-type counterparts suggested that the affinity was affected, as RegIIA is competing with ACh for the agonist binding site. In agreement with this notion, the RegIIA wash-off kinetics was also determined by the aforementioned residues. Recovery from block by RegIIA, differed significantly between human α3β2 and α3β4 nAChRs (1 min compared to 13-14 min), and the substitution of β2-β4 residues 59 and 113 switched the rate of recovery towards the opposite subtype (Cuny \textit{et al.}, 2016). Interestingly, residue 59 of the rat β subunits has also been identified as a determinant for α-conotoxin LvIA potency and wash-off kinetics (Luo \textit{et al.}, 2014). LvIA exhibited ~20-fold higher selectivity for rat α3β2 over α3β4. The rat β2[T59K] mutant further increased the peptide
activity by approximately ten-fold and considerably slowed the recovery from block by LvIA compared to wild-type α3β2 (Luo et al., 2014).

MD simulation studies provided further details on the interaction between human β2 and β4 residues 59 and 113 with RegIIA. RegIIA N9/β4 K59 contact (via hydrogen bond) is proposed to be critical for RegIIA activity at human α3β4 (Fig. 4A, E). In contrast, such contacts are not possible between RegIIA and human α3β2, as the side chain of the β2 corresponding residue, T59, is not long enough to interact with loop 2 of RegIIA (Fig. 4B, F). The increased potency and slower wash-off caused by the β2 T59K point mutation could then be explained by the gain of these additional hydrogen bonds, resulting in tighter binding of the peptide to the mutant nAChR. Computational modeling also revealed a crucial role of the long and positively charged side chain residue R113 in β4 for forming a H-bond with RegIIA (Fig. 4A, C). When the β4 R113 is introduced into the β2 backbone to replace the short chain-residue S113 (Fig. 4D), it forms H-bonds with the peptide similar to hα3β4 thereby stabilizing nAChR-RegIIA binding, improving its potency and significantly slowing its wash-off rate (Cuny et al., 2016). Other hα3β4 mutations, primarily within loop F, also decreased RegIIA sensitivity and increased ACh affinity, albeit less pronounced than the aforementioned residues 59 and 113. Therefore, it was concluded other residues within the agonist binding loops probably play an auxiliary role for the subtype selectivity of RegIIA by stabilizing the main interacting residues and shaping the topology of the agonist binding pocket (Cuny et al., 2016).

α-Conotoxin BuIA was also investigated for nAChR subtype selectivity and wash-off kinetics (Azam et al., 2005; Shiembob et al., 2006). In different species, including human, a slow recovery from BuIA block at α3β4 compared to significantly faster recovery at α3β2 was observed (Azam et al., 2005). Residues 59, 111 and 119 of rat β2 and β4 subunits were identified as being critical for the off-rate differences. Similar to findings with RegIIA, the off-rate of BuIA was slower at the β2[T59K] mutant compared to wild-type α3β2 (Shiembob et al., 2006).

Additional studies support the conclusion that residue 59 on the β subunit plays an important role for agonist and antagonist binding. Mutation β2 T59K has been shown to be critical for dihydro-β-erythroidine and neuronal bungarotoxin (agonists) sensitivity of α3β2 nAChR (Harvey et al., 1996), as well as affecting the affinity of ACh and nicotine (agonists) to α2β2 nAChR (Parker et al., 2001). The high selectivity of α-conotoxin MII for the α3β2 nAChR subtype (> 200-fold more active over other nAChR subtypes) was mapped to three sequence
segments using $\beta_2$-$\beta_4$ chimeras. Within the segments, $\beta_2$ residue T59 was identified as one factor determining the higher sensitivity of $\beta_2$ to MII compared to $\beta_4$ (Harvey et al., 1997).

Acetylcholine binding protein isolated from neurons of the giant sea slug *Aplysia californica* (Ac-AChBP) is homologous to the ligand-binding domains of nAChRs, and also pharmacologically similar. Since the time when its crystal structure was determined it has been well-proven as a valuable tool to study conotoxin-nAChR interactions. Co-crystal structures of $\alpha$-conotoxins in complex with Ac-AChBP have been reported for PnIA[A10L,D14K] (PDB code 2BR8), TxIA[A10L] (PDB code 2UZ6), ImI (PDB code 2C9T) BuIA (PDB code 4EZ1) and GIC (PDB code 5CO5) (Lin et al., 2016a).

Lin et al. compared their Ac-AChBP/GIC crystal structure with MD simulation models of GIC docking to $\alpha_3\beta_2$ and $\alpha_3\beta_4$, respectively, to obtain accurate toxin-receptor binding models and identify the key amino acid residues on the complementary receptor interface that appear to be responsible for selectivity of GIC for $\alpha_3\beta_2$ over $\alpha_3\beta_4$ (Lin et al., 2016b). The authors concluded that H5 and Q3 of GIC are the important residues interacting with the receptors. GIC H5 appeared to mainly interact with the principal binding interface of human $\alpha_3$, whereas on the complementary $\beta_2$ interface residues E61, V111, S113, S117 and F119 form a binding pocket for GIC Q13. In contrast, the non-conserved residues in human $\beta_4$ and most importantly R113 (labeled R115 in the aforementioned publication), lead to steric hindrance and even steric clashes with GIC Q13, resulting in overall strongly diminished binding affinity (Lin et al., 2016b). Note that $\beta_4$ R113 was shown to be a major determinant for the selectivity of RegIIA towards $\alpha_3\beta_4$ (as discussed above). This example shows again that common structural features of the receptors can be responsible for markedly different selectivity profiles of $\alpha$-conotoxins.

**Designing $\alpha$-conotoxin analogues to improve nAChR subtype selectivity**

Scientific advances such as MD simulation, co-crystal structures of $\alpha$-conotoxins with AChBP and functional studies with mutated receptors significantly improved our understanding of how these peptides can confer their remarkably unique selectivity profiles. The next step is to custom-design peptide analogues with improved affinity towards a specific receptor subtype while decreasing affinity at other subtypes to reduce off-target effects. Some studies provided the proof of concept that this is indeed possible. For example, as mentioned above, RegIIA gained enhanced selectivity for $\alpha_3\beta_4$ when its residues N11 and N12 are exchanged to alanine residues.
(Kompella et al., 2015b). Similarly, single amino acid changes in PnIA yielded improved potency and selectivity. Hogg et al. showed that PnIA[A10L] was an order of magnitude more potent than native PnIA at inhibiting ACh-evoked currents in rat parasympathetic neurons, albeit a maximal inhibition of only 45% of the peak current amplitude was observed compared to almost complete block by the native toxin. It was proposed that position 10 of PnIA influences potency and determines selectivity among α7 and other nAChR subtypes, including α3β2 and α3β4 (Hogg et al., 1999). Subsequent studies aimed at revealing the role of PnIA position 10 on potency and selectivity for the α7 and α3β2 nAChR subtypes (Hopping et al., 2014; Kasheverov et al., 2011). Electrophysiological evaluation of various PnIA[A10L] analogues revealed that hydrophobic residues in position 10 maintained potency at both subtypes whereas charged or polar residues abolished α7 binding thereby shifting selectivity towards α3β2 (Hopping et al., 2014). In a recent study, an α-conotoxin LsIA[R10F,N12L] double mutant was designed to specifically inhibit the α3β2 versus α7 nAChR subtype (Abraham et al., 2017). The wild-type LsIA is about 150 times more potent at α7 than α3β2, whereas LsIA[R10F,N12L] possesses >250-fold selectivity for α3β4 over α7 nAChR (Abraham et al., 2017).

In addition to the aforementioned targeted approaches to improve α-conotoxin selectivity based on known structural data, large-scale synthetic combinatorial screens are also a powerful tool to discover peptide analogues with improved pharmacological properties. A synthetic combinatorial library derived from α-conotoxin BuIA sequence revealed eleven analogues with inhibitory activity at the α3β4 nAChR. One of these analogues, termed TP-2212-59, is one of the most potent and selective α3β4 antagonists known to date, with a calculated IC_{50} of 2.3 nM at α3β4 and more than 1000-fold less activity at α3β2, and α7 subtypes (Chang et al., 2014).

Conclusions
Recent findings with RegIIA, AuIB and other α-conotoxins confirm that subtype-selective nAChR antagonists often work through a common mechanism, by interacting with the same structural components and sites on the receptor. In future, with increasing knowledge about the interactions necessary for selective and potent antagonist binding, new pharmacological probes can be custom-designed to target specific nAChR subtypes. These probes will not only help to elucidate the physiological roles of non-CNS nAChRs but also to advance the discovery of novel drug candidates to treat diseases in which a particular nAChR subtype is the underlying cause.
Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan et al., 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/2016 (Alexander et al., 2015).

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Conflicting interests

The authors have no conflict of interest to declare
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**Fig. 1.** Crystal structure of human α4β2 nAChR with bound nicotine molecules (PDB ID: 5KXI) (Morales-Perez et al., 2016). (A) Side view. Two nicotine molecules (NCT, orange) are bound at the extracellular domain of the receptor. Arrow and orange dots indicate the direction of cation movement. Dashed orange line indicates the plasma membrane. The α4 and β2 subunits are colored green and cyan, respectively. (B) Magnification of the nicotine binding site (dotted box from A), formed at the interface of α4 and β2 subunits. ACh, the endogenous nAChR agonist, occupies the same site. The binding pocket consists of loops A, B and C of the α4 subunit, indicated by the arrows, and loops D, E and F of the β2 subunit (not shown). (C) Top view of the α4β2 nAChR pentamer. Note the central ion-conducting pore.
**Fig. 2.** Sequence and structure of α3β2 and α3β4 nAChR-targeting α-conotoxins. (A) Sequence alignment of 13 α-conotoxins that antagonize α3β2 and α3β4 nAChRs. Cysteine residues C_I- C_IV (grey columns) form the disulfide bridges between C_I-C_III and C_II-C_IV (black lines) in native α-conotoxins. * indicates C-terminal amidation. γ in GID sequence refers to γ-carboxyglutamate residue. B and Z in the TP-2212-59 sequence refer to 2-aminobutyric acid and norvaline, respectively. (B and C) Structures of α-conotoxins AuIB and RegIIA, respectively.
Fig. 3. MD simulation models of AuIB (magenta) binding at human α3β4 and α3β2 nAChRs. (A) Residue F9 of AuIB is oriented outside the WLK pocket (residues W57, L119 and K59) of human β4 (cyan), thereby preventing direct interaction. (B) AuIB F9 is oriented in close proximity to W57 at human β2 (pink) and can form the crucial π-π interaction, but the short and hydrophilic side chain of β2 T59 is ineffective at stabilizing AuIB F9 and forming a binding pocket.
**Fig. 4.** Molecular docking models illustrating the binding of RegIIA to human $\alpha_3\beta_4$ (A) and $\alpha_3\beta_2$ (B) nAChRs, respectively. Several H-bonds (dotted lines) with threshold distance 3.2 Å are formed between pairwise interacting residues of different loops or $\beta$-sheets, thereby affecting their local conformation or dynamics, which in turn affects the binding of RegIIA. The $\alpha_3(\pm)$ interface is shown in green, $\beta_2(\pm)$ in pink, $\beta_4(\pm)$ in cyan and RegIIA in orange. Residues nearby the agonist binding site that are essential for the interaction with RegIIA are shown as licorice models. Residues from the receptor and RegIIA are labelled using normal and italic fonts, respectively. Non-conserved residues forming H-bonds are highlighted with circles. (C-F) Magnification of the interaction sites in $\alpha_3\beta_4$ (C, E) and $\alpha_3\beta_2$ (D, F). The neutral and short $\beta_2$ subunit side chain residues T59 and S113 cannot interact with backbone atoms of N9 and N11 of RegIIA. In contrast, the long positively charged side chains of K59 and R113 of $\beta_4$ form H-bonds with RegIIA N9 and C16, and N11 residues, respectively.
Table 1: Tissue distribution\(^a\) of α3, α5, α6, β2 and β4 nAChR subunits in the peripheral nervous system (PNS) and non-neuronal cells.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>nAChR subunit</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PNS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autonomic ganglia</td>
<td>α3, α5, β2, β4</td>
<td>(Bibevski et al., 2000; Campanucci et al., 2010; Del Signore et al., 2004; Jensen et al., 2005; Millar et al., 2009; Park et al., 2006; Purnyn et al., 2004; Rassadi et al., 2005; Rudchenko et al., 2014; Wang et al., 2005; Xu et al., 1999a; Xu et al., 1999b)</td>
</tr>
<tr>
<td>Adrenal chromaffin cells</td>
<td>α3, α5, β2, β4</td>
<td>(Campos-Caro et al., 1997; Sala et al., 2008; Tachikawa et al., 2001)</td>
</tr>
<tr>
<td>Dorsal root ganglia</td>
<td>α3, α6, β2, β4</td>
<td>(Fucile et al., 2005; Genzen et al., 2001; Hone et al., 2012; Khan et al., 2003; Smith et al., 2013; Spies et al., 2006; Zhang et al., 2015)</td>
</tr>
<tr>
<td>Enteric ganglia</td>
<td>α3, α5, β2, β4</td>
<td>(Foong et al., 2015; Galligan et al., 2004; Glushakov et al., 2004; Zhou et al., 2002)</td>
</tr>
<tr>
<td>Nodose (vagal) ganglia</td>
<td>α3, α5, β2, β4</td>
<td>(Mao et al., 2006)</td>
</tr>
<tr>
<td><strong>Non-neuronal cells</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Keratinocytes</td>
<td>α3, α5, β2, β4</td>
<td>(Chernyavsky et al., 2004; Grando et al., 1995; Hagforsen, 2007; Kurzen et al., 2004; Zia et al., 2000)</td>
</tr>
<tr>
<td>Lung epithelial cells</td>
<td>α3, α5, β2, β4</td>
<td>(Conti-Fine et al., 2000; Maus et al., 1998; Proskocil et al., 2004; Sekhon et al., 2005; Zia et al., 1997)</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>α3, α5, β2, β4</td>
<td>(Bruggmann et al., 2003; Hawkins et al., 2005; Heeschen et al., 2002; Macklin et al., 1998; Moccia et al., 2004)</td>
</tr>
<tr>
<td>Astrocytes</td>
<td>α3, α5, β2, β4</td>
<td>(Graham et al., 2003)</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>α3, α5, β2, β4</td>
<td>(Hiemke et al., 1996; Skok et al., 2007)</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>α3, β2, β4</td>
<td>(Benhammou et al., 2000; Blanchet et al., 2007)</td>
</tr>
<tr>
<td>Mast cells</td>
<td>α3, α5, β2?, β4?</td>
<td>(Kindt et al., 2008)</td>
</tr>
<tr>
<td>Macrophages</td>
<td>α3, β2?, β4?</td>
<td>(Yang et al., 2016)</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>α3, α5, β2, β4</td>
<td>(Arredondo et al., 2003)</td>
</tr>
</tbody>
</table>

\(^a\) determined by protein and/or functional expression of α3-containing nAChRs
Table 2: Selected α-conotoxins that exhibit inhibitory activity at α3β2 and α3β4 nAChRs expressed in *Xenopus* oocytes.

<table>
<thead>
<tr>
<th>α-Conotoxin</th>
<th>Frame-work</th>
<th>(^a)IC(_{50}) (nM) at recombinant nAChRs</th>
<th>(^b)Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ImI</em> &lt;br&gt;<em>C. imperialis</em></td>
<td>4/3</td>
<td>40.8 (h) &lt;br&gt;(Ellison <em>et al.</em>, 2004)</td>
<td>3390 (h) &lt;br&gt;(Ellison <em>et al.</em>, 2004)</td>
</tr>
<tr>
<td><em>BuIA</em> &lt;br&gt;<em>C. bullatus</em></td>
<td>4/4</td>
<td>5.72</td>
<td>27.7</td>
</tr>
<tr>
<td>TP-2212-59 &lt;br&gt;(synthetic)</td>
<td>4/4</td>
<td>&gt; 10,000</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt; 30,000 (h) &lt;br&gt;(Cuny <em>et al.</em>, 2016)</td>
<td>&gt; 30,000 (h) &lt;br&gt;(Cuny <em>et al.</em>, 2016)</td>
</tr>
<tr>
<td><em>TxID</em> &lt;br&gt;<em>C. textile</em></td>
<td>4/6</td>
<td>&gt; 10000</td>
<td>12.5</td>
</tr>
<tr>
<td><em>MII</em> &lt;br&gt;<em>C. magus</em></td>
<td>4/7</td>
<td>1.7 &lt;br&gt;(Dowell <em>et al.</em>, 2003)</td>
<td>&gt; 200</td>
</tr>
<tr>
<td><em>PIA</em> &lt;br&gt;<em>C. purpurascens</em></td>
<td>4/7</td>
<td>74.2</td>
<td>518</td>
</tr>
<tr>
<td><em>RegIIA</em> &lt;br&gt;<em>C. regius</em></td>
<td>4/7</td>
<td>33 &lt;br&gt;(Franco <em>et al.</em>, 2012)</td>
<td>97 &lt;br&gt;(Franco <em>et al.</em>, 2012)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>132.4 (h) &lt;br&gt;(Cuny <em>et al.</em>, 2016)</td>
<td>45.6 (h) &lt;br&gt;(Cuny <em>et al.</em>, 2016)</td>
</tr>
<tr>
<td><em>GIC</em> &lt;br&gt;<em>C. geographus</em></td>
<td>4/7</td>
<td>1.1 (h)</td>
<td>755 (h)</td>
</tr>
<tr>
<td><em>GID</em> &lt;br&gt;<em>C. geographus</em></td>
<td>4/7</td>
<td>3.1</td>
<td>&gt;10000</td>
</tr>
<tr>
<td><em>PnIA</em> &lt;br&gt;(synthetic)</td>
<td>4/7</td>
<td>9.56 &lt;br&gt;(Luo <em>et al.</em>, 1999)</td>
<td>&gt;1000 &lt;br&gt;(Everhart <em>et al.</em>, 2003)</td>
</tr>
<tr>
<td><em>LsIA</em> &lt;br&gt;<em>C. limpusi</em></td>
<td>4/7</td>
<td>10</td>
<td>&gt; 1000</td>
</tr>
<tr>
<td><em>LvIA</em> &lt;br&gt;<em>C. lividus</em></td>
<td>4/7</td>
<td>8.67 &lt;br&gt;(h)</td>
<td>148</td>
</tr>
</tbody>
</table>
Most α-conotoxins listed were tested on a range of nAChR subtypes, however, only IC₅₀ values for subtypes covered in this review are shown. Refer to cited literature for full details.

*If not indicated otherwise, IC₅₀ values were obtained at rat nAChRs. References to individual values indicate the publication in which the data were reported. (h) human.

*Reference in which the α-conotoxin was first described.

IC₅₀ values were obtained with the synthetic analogue PnIA[sTy15Y], which is commonly referred to as PnIA, albeit amino acid 15 differs from native PnIA of *C. pennaceus*. 
<table>
<thead>
<tr>
<th></th>
<th>C_{I}</th>
<th>C_{II}</th>
<th>C_{III}</th>
<th>C_{IV}</th>
</tr>
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<tbody>
<tr>
<td>RegIIA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>GCC</td>
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<tr>
<td>MII</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>GCC</td>
</tr>
<tr>
<td>GIC</td>
<td>-</td>
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<td>-</td>
<td>GCC</td>
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<tr>
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<td>GCC</td>
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<tr>
<td>LvlIA</td>
<td>-</td>
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<td>-</td>
<td>GCC</td>
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<tr>
<td>LslIA</td>
<td>-</td>
<td>-</td>
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<td>GCC</td>
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<tr>
<td>PIA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>GCC</td>
</tr>
<tr>
<td>GID</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>GCC</td>
</tr>
<tr>
<td>AulB</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>GCC</td>
</tr>
<tr>
<td>TxlID</td>
<td>-</td>
<td>-</td>
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<td>GCC</td>
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<tr>
<td>BulA</td>
<td>-</td>
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<td>TP-2212-59</td>
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<td>GCC</td>
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<tr>
<td>lml</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>GCC</td>
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</table>

![AulB](image1.png)

![RegIIA](image2.png)