

Novel structural determinants of single channel conductance and ion selectivity in 5-hydroxytryptamine type 3 and nicotinic acetylcholine receptors

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Nicotinic acetylcholine (nACh) and 5-hydroxytryptamine type 3 (5-HT₃) receptors are cation-selective ion channels of the pentameric ligand-gated ion channel (pLGIC) super-family. Multiple lines of evidence adduced over the last 30 years indicate that the lining of the channel of such receptors is formed by the α -helical second transmembrane (TM2) domain and flanking sequences contributed by each of the five subunits present within the receptor complex. Specific amino acid residues within, and adjacent to, the TM2 domain influence single channel conductance, ion selectivity, and other aspects of receptor function that include gating and desensitization. However, more recent work has revealed important structural determinants of single channel conductance and ion selectivity that are not associated with the TM2 domain. Direct experimental evidence indicates that the intracellular domain of eukaryotic pLGICs, in particular a region of the loop linking TM3 and TM4 termed the membrane-associated (MA) stretch, exerts a strong influence upon ion channel biophysics. Moreover, recent computational approaches, complemented by experimentation, implicate the extracellular domain as an additional important determinant of ion conduction. This brief review describes how our knowledge of ion conduction and selectivity in cation-selective pLGICs has evolved beyond TM2.

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Abbreviations 5-HT, 5-hydroxytryptamine; ACh, acetylcholine; AChBP, acetylcholine binding protein; ECD, extracellular domain; ICD, intracellular domain; MA stretch, membrane-associated stretch; MD, molecular dynamics; MTS, methanethiosulphonate; pLGIC, pentameric ligand-gated ion channel; TM, transmembrane; γ , single channel conductance.

Introduction

5-Hydroxytryptamine type 3 (5-HT₃) and nicotinic acetylcholine (nACh) receptors, along with a much less extensively studied zinc-activated channel (ZAC), form the excitatory, cation selective, members of the pentameric ligand-gated ion channel (pLGIC)

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work at the Centre for Neuroscience at the University of Dundee. They have a particular interest in the physiological and pharmacological properties of ligand-



gated ion channels of the 'Cys-loop receptor' family. Much of their work focuses on how the structure of such receptors relates to their function. Tim Hales, their long-term collaborator in this enterprise has recently moved from George Washington University to join them in Dundee.

superfamily in eukaryotes (Barnes *et al.* 2009; Millar & Gotti, 2009). The functionally opposing inhibitory, anion selective channels are the γ -aminobutyric acid type A (GABA_A) and strychnine-sensitive glycine receptors (Olsen & Sieghart, 2008; Lynch, 2009). Each of the five subunits of the eukaryotic pLGICs consists of three functional modules: (i) an extracellular domain (ECD) harbouring the ligand binding site, (ii) four α -helical transmembrane (TM) domains, the second of which forms the lining of the ion channel, and (iii) an intracellular domain (ICD), composed of a large loop between the TM3 and TM4 domains and a short sequence linking the TM1 and TM2 domains (Fig. 1). The large loop is essentially absent from the prokaryotic pLGICs of *Gloeobacter violaceus* (i.e. GLIC) and *Erwinia chrysanthemi* (i.e. ELIC) (Hilf & Dutzler, 2009).

A detailed picture of the three dimensional structure of the pLGIC subunits has been revealed by a number of advances. Firstly, the crystal structure of the molluscan acetylcholine binding protein (AChBP), which is a water soluble pentameric homologue of the ECD of the nACh receptor, in complex with several nicotinic receptor ligands, has been determined (Rucktooa *et al.* 2009). The latter has permitted homology modelling of the occupied ligand binding domain of the major families of eukaryotic pLGICs. Secondly, the structural organisation of almost the entirety of the nACh receptor of *Torpedo*

marmorata has been solved, at 4 Å resolution, through progressive improvements in cryo-electron microscopy and image reconstruction (Unwin, 2005). Importantly, this structure adds details of the organisation of the TM domains to the information on the ECD gleaned from AChBP. Unfortunately, a substantial portion of ICD of the nACh receptor, which is predicted to be mostly unfolded (Kukhtina *et al.* 2006), remains to be characterised structurally. Nonetheless, α -helical structures are predicted to occur within two segments of the loop linking TM3 and TM4 (Kukhtina *et al.* 2006) and one, dubbed the MA (or HA) stretch (Finer-Moore & Stroud, 1984), is resolved in the refined model of the *Torpedo* nACh receptor (Unwin, 2005). The influence of the MA stretch upon receptor biophysics will be emphasised in this review. Finally, the X-ray structures of GLIC and ELIC have provided the first high resolution structures of pLGIC superfamily members. Importantly, a comparison of the structures of GLIC, obtained in the conducting state, and ELIC, observed in the non-conducting state, provides insight into the gating process (Hilf & Dutzler, 2009).

The conduction pathway

The most complete description of the journey of an ion from the extracellular to intracellular media *via* eukaryotic pLGICs is provided by the *Torpedo* nACh receptor (Miyazawa *et al.* 2003; Unwin, 2005). In outline, ions enter the conduction pathway through the extracellular vestibule, which has an inner diameter of approximately 20 Å and is approximately 60 Å long. Thereafter they translocate to the narrower transmembrane pore that is lined by the five TM2 α -helices and which contains the gate of the channel. At the intracellular aspect of the membrane, the ions emerge into a vestibule perforated by five narrow fenestrations ('portals') that form an obligate pathway through which ions must flux. Such portals are framed laterally by α -helices corresponding to the MA stretch of adjacent subunits and at their roof by the TM1–TM2 loop. The α -helices project interiorly into the cytoplasm and converge on the central axis of the receptor in the manner of an inverted tepee (Miyazawa *et al.* 2003; Unwin, 2005). Thus, in this model of eukaryotic pLGIC structure, the cytoplasmic vestibule presents five parallel conduction pathways, rather than a single, central conduit. Evidently, the prokaryotic pLGICs lack this structural sophistication raising questions about its purpose in the evolutionarily more advanced channels.

A seminal mutagenesis study of the nACh receptor of *Torpedo californica* identified three clusters of negatively charged residues that bracket the channel-lining TM2 segment as crucial determinants of single channel conductance (γ), namely the extracellular (20'), inner

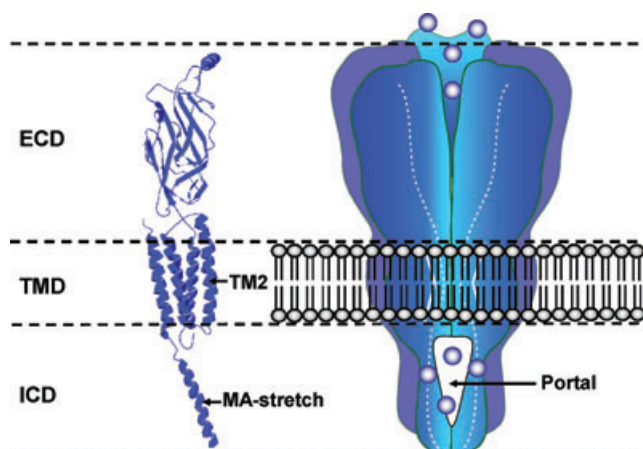


Figure 1. The architecture of the eukaryotic members of the pentameric ligand-gated ion channel superfamily

Left, a homology model of a human 5-HT_{3A} receptor subunit constructed by Hales *et al.* (2006) from the 4 Å resolution data of the *Torpedo* nACh receptor (Unwin, 2005). The locations of the channel lining TM2 domain and MA stretch that frames the lateral aspects of the portals are indicated. Right, schematic illustration of the gross organisation of such receptors indicating the intracellular location of the portals that have recently been shown to contribute to the permeation pathway (Kelley *et al.* 2003) and which form an obligate route for ion flux. Five such portals are located at the interfaces of the subunits comprising the receptor. Abbreviations: ECD, extracellular domain; TMD, transmembrane domain; ICD, intracellular domain; MA, membrane-associated.

(−1′) and cytoplasmic (−4′) rings of charge (Imoto *et al.* 1988; see Fig. 2). Subsequently, the importance of TM2 and the flanking rings of charge in ion conduction and selectivity has become axiomatic for all eukaryotic pLGICs, including the anion selective GABA_A and glycine receptors (in which the charged rings occur at the 0′ and 19′ locations and are net positive) (Keramidas *et al.* 2004; Jensen *et al.* 2005; Peters *et al.* 2005). However, more recent studies have revealed that important determinants of γ and ion selectivity also reside within the ICD and the ECD of 5-HT₃ and nACh receptors (Kelley *et al.* 2003; Hales *et al.* 2006; Deeb *et al.* 2007; Hansen *et al.* 2008; Livesey *et al.* 2008). The influence of specific amino acid residues within these domains upon the biophysical properties of nACh and 5-HT₃ receptors is discussed below.

The differential single channel conductance (γ) of homomeric 5-HT_{3A} and heteromeric 5-HT_{3AB} receptors led to the localization of a major determinant of γ to the ICD

Genes encoding five 5-HT₃ receptor subunits (5-HT_{3A–E}) have been identified in humans (Karnovsky *et al.* 2003; Niesler *et al.* 2003), but only homomeric 5-HT_{3A} (Brown *et al.* 1998) and heteromeric 5-HT_{3AB} (Davies *et al.* 1999) receptors have been studied in detail. Note that the receptor and subunit nomenclature employed in this article conforms to recent recommendations issued by NC-IUPHAR (Collingridge *et al.* 2009). Human 5-HT_{3A} and 5-HT_{3AB} receptors display only modest differences in their ligand binding profile when probed by conventional 5-HT₃ receptor ligands (Brady *et al.* 2001) but differ markedly in their biophysical properties including γ , relative permeability to divalent cations and rectification at the single channel level (Brown *et al.* 1998; Davies *et al.* 1999; Mochizuki *et al.* 1999; Kelley *et al.* 2003). Most notably, the γ (~16 pS) of the 5-HT_{3AB} receptor in outside-out membrane patches excised from HEK-293 cells transfected with cDNAs encoding the 5-HT_{3A} and 5-HT_{3B} subunits is much greater than that of the recombinant 5-HT_{3A} receptor (0.4–1.0 pS) inferred by fluctuation analysis of macroscopic current responses (Brown *et al.* 1998; Davies *et al.* 1999; Kelley *et al.* 2003; Deeb *et al.* 2007). The structural basis of the anomalously low γ of the 5-HT_{3A} receptor does not reside within the TM2 domain, which exhibits a high degree of conservation to that of the high conductance $\alpha 7$ nACh receptor (reviewed by Peters *et al.* 2005; see also Fig. 2). There is no doubt that TM2 domains form the lining of the 5-HT_{3A} receptor ion channel: the mutation E-1′A, thus neutralising the inner ring of charge, results in a channel that does not discriminate between monovalent cations and anions ($P_{Na}/P_{Cl} = 0.89$) and when combined with the mutation S19′R produces

a channel with mild anion selectivity ($P_{Na}/P_{Cl} = 0.37$) (Thompson & Lummis, 2003). Similarly, as found for the $\alpha 7$ nACh receptor (Galzi *et al.* 1992), the mutations V13′T and E-1′A, coupled with the insertion of proline into the TM1–TM2 loop, generate an anion selective channel ($P_{Na}/P_{Cl} = 0.08$) (Gunthorpe & Lummis, 2001). More recently, we found that neutralisation of the extracellular ring of charge by the mutation D20′A markedly suppressed the relative permeability of Ca²⁺, but had no influence upon monovalent cation *versus* anion selectivity (Livesey *et al.* 2008).

To identify the domain of the 5-HT_{3B} receptor subunit that confers greatly enhanced γ on 5-HT_{3AB} over 5-HT_{3A} receptors, we constructed a series of human 5-HT_{3A}/5-HT_{3B} subunit chimaeras, expressed them in tsA-201 cells either in isolation or in combination with wild-type 5-HT_{3A} subunits, and analysed γ initially by fluctuation analysis (Kelley *et al.* 2003). This approach, schematically illustrated in Fig. 3, identified the intracellular TM3–TM4 loop as harbouring a crucial determinant of γ . Finer inspection of the loop by less extensive substitution of 5-HT_{3A} by 5-HT_{3B} subunit sequence within homomeric receptors revealed that replacement of an 18 amino acid sequence within the amphipathic MA stretch increased γ by approximately 29-fold (Kelley *et al.* 2003). A striking feature of all orthologous 5-HT_{3A} subunit sequences is the presence of three arginine residues (R432, R436 and R440 of the human sequence) with a periodicity that places them on the same face of an α -helical structure (Fig. 3). Such a motif is absent from any other vertebrate cation selective pLGIC subunit. The collective mutation of these residues to their aligned counterparts within the 5-HT_{3B}

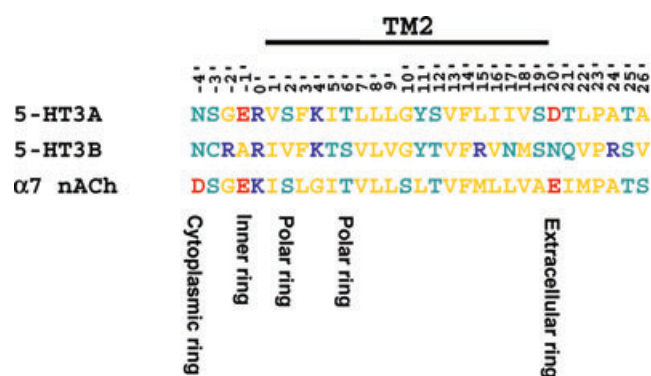


Figure 2. Sequence alignment of the human 5-HT_{3A}, 5-HT_{3B} and $\alpha 7$ nACh subunit across the TM2 domain and flanking sequences

Note that structural elements identified by Imoto *et al.* (1988) as important determinants of γ (i.e. the extracellular and inner rings of charge) are conserved between 5-HT_{3A} and $\alpha 7$ nACh subunits, but are absent from the 5-HT_{3B} subunit. Within the alignment basic, acidic, polar and non-polar residues are colour coded blue, red, green and yellow, respectively.

subunit (i.e. R432Q, R436D, R440A) enhanced γ by a factor comparable to that caused by the 18 amino acid insertion of 5-HT₃B subunit sequence described above. An analysis of single amino acid substitutions revealed the R436D mutation to have the most pronounced impact upon γ (Kelley *et al.* 2003). We substantiated the results of fluctuation analysis by single channel recordings performed on outside-out membrane patches and note that whilst the former method consistently underestimates γ determined directly, there is nonetheless an excellent correlation between the data obtained by the two methods (Hales *et al.* 2006). For convenience of description, we will henceforth refer to the 5-HT₃A R432Q, R436D, R440A mutant as the 5-HT₃A (QDA) receptor. Under standard recording conditions, the γ of the human 5-HT₃A (QDA) receptor is 22 pS, or 36 pS, when determined by fluctuation analysis of whole cell currents, or single channel recording from outside-out membrane patches, respectively (Kelley *et al.* 2003; Hales *et al.* 2006).

The dramatic increase in γ exhibited by the 5-HT₃A (QDA) receptor has been confirmed by at least two other groups who performed mutagenesis upon the mouse, rather than human, 5-HT₃A subunit. (Reeves *et al.* 2005; Bouzat *et al.* 2008). Moreover, the replacement of the entire intracellular loop linking TM3 and TM4 of the mouse 5-HT₃A subunit by the short heptapeptide linker of GLIC results in a channel (termed 5-HT_{3A}-glvM3M4) that is apparently fully functional and has a γ (i.e. 43 pS; Jansen *et al.* 2008) remarkably similar to that of the human 5-HT₃A (QDA) receptor (i.e. 38–42 pS; Reeves *et al.* 2005; Livesey *et al.* 2008) under approximately equivalent ionic conditions. Jansen *et al.* (2008) therefore speculate ‘... that no additional features in the whole M3M4 loop besides the

three arginines are involved in limiting the 5-HT_{3A} single channel conductance ...’. An alternative, and in our view more likely, interpretation is that the similar values of γ found for the 5-HT₃A (QDA) and 5-HT_{3A}-glvM3M4 receptor constructs reflect a common rate limiting step in ion conduction that emerges when the constraint imposed by the whole TM3–TM4 loop, or the triple arginine motif, is removed.

A homology model of the human 5-HT₃A receptor (constructed from the atomic scale model of the *Torpedo* nACh receptor) provides a qualitative glimpse of how the MA stretch R432, R436 and R440 residues might collectively influence γ so profoundly (Hales *et al.* 2006). Inspection of the model depicted in Fig. 4 reveals the three arginine residues to be positioned within the intracellular portals through which ions must pass to enter, or leave, the cytoplasm. The dimensions of the portals are such that the positively charged and voluminous R432, R440 and particularly R436 residues could act as both steric and electrostatic impediments to cation flux (Fig. 4). It must be acknowledged that the orientations of the residue side chains are not known precisely and that the model is based on the *Torpedo* nACh receptor in the *closed* state. However, the following section reviews additional data that support the model.

R436 of the human 5-HT₃A receptor is a key residue in determining γ and divalent versus monovalent cation selectivity

The solitary substitution R436D within the MA stretch of the human 5-HT₃A receptor increases γ from a value

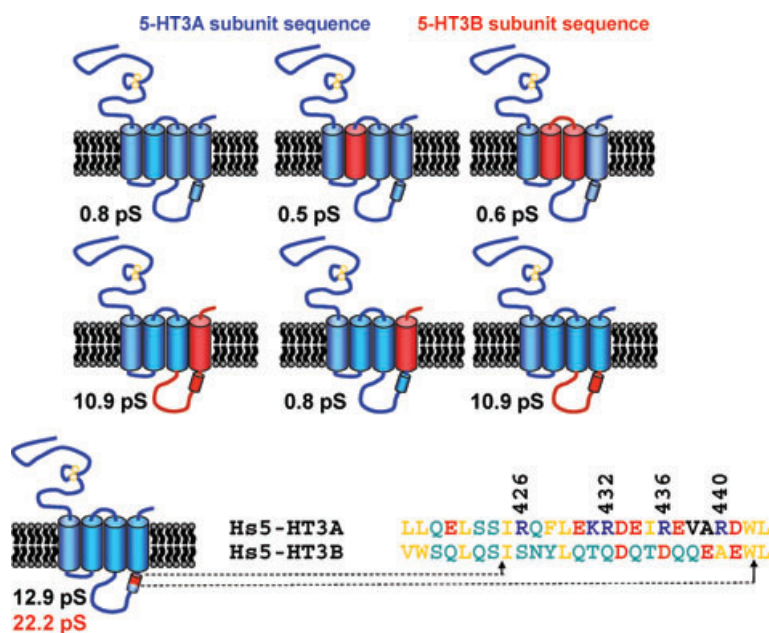


Figure 3. Summary of the experimental approach that resulted in the identification of the MA stretch as a component of the ion permeation pathway

Representative 5-HT₃A/5-HT₃B subunit chimaeras used by Kelley *et al.* (2003) are shown schematically with human 5-HT₃A and 5-HT₃B sequences depicted in blue and red, respectively. Single channel conductances (γ , estimated by fluctuation analysis) for the chimaeric constructs co-expressed with the wild-type 5-HT₃A subunit in tsA-201 cells are given in each panel. Note that a second γ value (in red) indicates that the construct functioned as a homoligomer obviating the need for co-expression with the wild-type 5-HT₃A subunit. The final chimaera examined (bottom row) identified an 18 amino acid sequence within the α -helical MA stretch as a crucial determinant of γ . A sequence alignment across this region indicates the location of the R432, R436 and R440 residues of the human 5-HT₃A subunit and their human 5-HT₃B subunit counterparts. The collective substitution of the latter into the 5-HT₃A sequence increased γ by approximately 40-fold in comparison to the wild-type 5-HT₃A receptor (Hales *et al.* 2006). Within the alignment basic, acidic, polar and non-polar residues are colour coded blue, red, green and yellow, respectively.

of 0.4–1 pS to approximately 9 pS. Similarly, the mutation R440A has a substantial effect upon γ , which increases to approximately 5 pS, but the substitution R432Q has little influence (Hales *et al.* 2006). The pronounced influence of R436 provided an impetus to evaluate this locus more comprehensively by the use of a cysteine residue that replaced the arginine to form a substrate for covalent modification by reactive methanethiosulphonate (MTS) reagents (Deeb *et al.* 2007). The R436C mutation elevated γ (to approximately 8 pS), but a superior window for evaluating reduction, or enhancement, of γ by MTS reagents was provided by the 5-HT_{3A} R3432Q, R436C, R440A (5-HT_{3A} (QCA)) receptor construct which was found to have a γ of approximately 18 pS (Deeb *et al.* 2007). MTS reagents were selected on the basis of charge and size in an attempt to determine the contribution of electrostatic and steric factors to the detrimental effect of R436 upon γ . In brief, both volume and charge at the 436 position influence γ , since an increase in volume, but no addition of charge, provided by modification of the cysteine residue

by neutral propyl-MTS reduces γ , but not to the same extent as 2-aminoethyl-MTS, which adds similar volume, but additionally positive charge. Negatively charged 2-carboxyethyl-MTS and 2-sulphonatoethyl-MTS both increase γ , despite increasing side chain volume at the 436 position (Deeb *et al.* 2007). The data are consistent with the side chain orientation of R436 predicted in the homology model of the 5-HT_{3A} receptor in which the positively charged guanidinium group projects into the portal.

The electrostatic environment provided by the triplet arginine motif could potentially impact upon the ion selectivity of the 5-HT_{3A} receptor. However, the 5-HT_{3A} (QDA) receptor retains perfect selectivity for mono-valent cations over anions (Livesey *et al.* 2008). Clearly, the residues identified by Thompson & Lummis (2003; see above) dominate over any influence of the portal region, a conclusion reinforced by the observation that the 5-HT_{3A}-glvM3M4 construct that lacks the large intracellular loop is also strongly cation selective (Jansen *et al.*

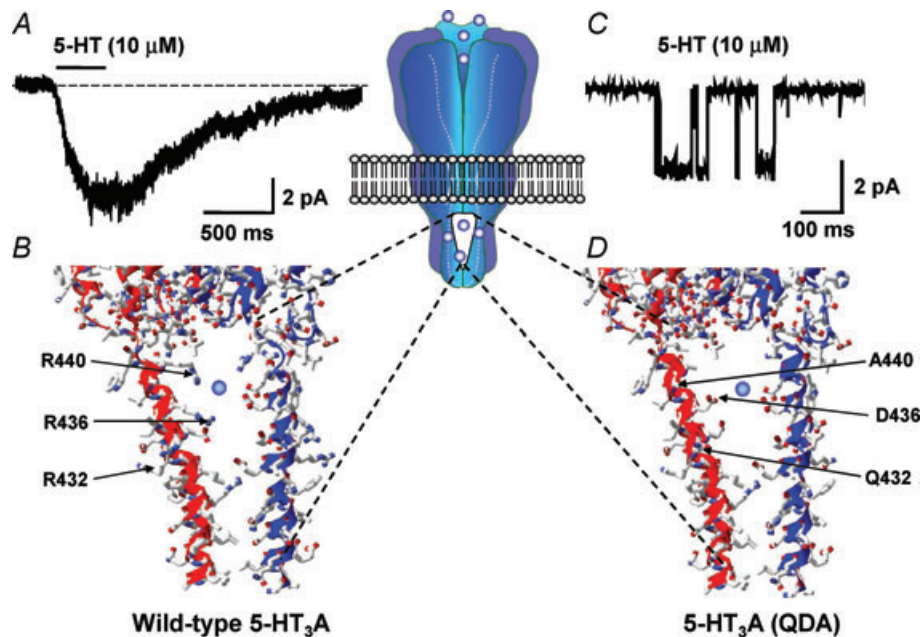


Figure 4. The influence of amino acid residues within the MA stretch upon the γ of the human 5-HT_{3A} receptor

A, inward current response evoked by 5-HT applied to an outside-out membrane patch excised from a HEK-293 cell expressing the human homomeric 5-HT_{3A} receptor. The patch was voltage-clamped at a holding potential of -60 mV. Note the absence of discernable single channel current events. **B**, homology model, constructed as described by Hales *et al.* (2006) from the 4 Å resolution structure of the *Torpedo* nACh receptor, of an intracellular portal of the homomeric 5-HT_{3A} receptor illustrating the arginine residues that collectively influence γ . Note that the side chains of R436 and R440 are predicted to face into the portal to impede cation ion flux. **C**, single channel currents evoked by 5-HT applied to an outside-out membrane patch excised from a tsA-201 cell expressing the human 5-HT_{3A} (QDA) receptor construct. The patch was voltage-clamped at a holding potential of -80 mV. **D**, homology model of an intracellular portal subsequent to the mutations R432Q, R436D and R440A (i.e. 5-HT_{3A} (QDA)). Note that reduced steric hindrance to ion flux is predicted, together with a local electrostatic potential more conducive to ion permeation. The central inset schematically illustrates the location of the portals within the receptor.

2008). However, divalent cations, such as Ca^{2+} , would experience a particularly strong repulsive Coulombic force emanating from the arginine triplet and consistent with this we found the 5-HT_{3A} (QDA) construct to have a much greater relative permeability to Ca^{2+} ($P_{\text{Ca}}/P_{\text{Cs}} = 3.7$) than the wild-type receptor ($P_{\text{Ca}}/P_{\text{Cs}} = 1.4$) (Livesey *et al.* 2008). The influence of the individual arginine residues upon relative permeability to Ca^{2+} parallels the findings for γ , since the R436D and R440A mutations increase $P_{\text{Ca}}/P_{\text{Cs}}$ to 3.1 and 2.3, respectively, whereas the R432Q substitution is essentially without effect (Livesey *et al.* 2008). However, such increases in relative permeability to Ca^{2+} do not appear to result in an enhanced fractional current carried by the divalent (Pf %). Noam *et al.* (2008) found that the Pf % value for the wild-type 5-HT_{3A} receptor (4.1) was in fact higher than that for the 5-HT_{3A} (QDA) receptor construct (1.8). The latter value approximates to that found for the heteromeric 5-HT_{3AB} receptor (i.e. 2.1; Noam *et al.* 2008).

Residues within the MA stretch influence voltage-dependent block of the human 5-HT_{3A} receptor by extracellular Ca^{2+}

Intracellular dialysis with a variety of phosphate-containing compounds renders the human 5-HT_{3A} receptor susceptible to voltage-dependent blockade by extracellular Ca^{2+} , independent of phosphorylation (Noam *et al.* 2008). Specifically, cytoplasmic phosph[on]ate compounds including ATP, GTP, CTP, UTP PPP_i and particularly PP_i and P_i induce a region of negative slope conductance in the macroscopic current–voltage (I – V) relationship to 5-HT at negative holding potentials, conditional upon the presence of extracellular Ca^{2+} (Noam *et al.* 2008). In the absence of intracellular phosph[on]ates the I – V relationship is inwardly rectifying, irrespective of the presence or absence of Ca^{2+} in the extracellular medium (Noam *et al.* 2008). This finding apparently reconciles divergent reports in the literature where Ca^{2+} -induced blockade of macroscopic currents mediated by 5-HT₃ receptors has been shown to be either voltage independent (e.g. Brown *et al.* 1998; Hu & Lovinger, 2005), or voltage dependent (e.g. Kawa, 1994; van Hooft & Wadman, 2003), the latter studies having been conducted upon cells dialysed with ATP. Significantly, intracellular phosphosph[on]ates do not confer voltage-dependent block by Ca^{2+} upon the 5-HT_{3A}(QDA) receptor (Noam *et al.* 2008), although we have shown that the γ of this construct is reduced by extracellular Ca^{2+} in a concentration-dependent and voltage-independent manner (Livesey *et al.* 2008). Hydrogen-bonded and salt bridged complexes between the guanidinium head group of arginine and phosph[on]ates, at concentrations that

are biologically relevant, are extensively documented (Schug & Lindner, 2005). Whether such an interaction is involved in the genesis of voltage-dependent block by Ca^{2+} that is induced by intracellular phosph[on]ates is unknown.

The MA stretch also influences the γ of nACh $\alpha 4\beta 2$ receptors

To assess whether residues within the MA stretch exert an influence upon the γ of other cation-selective pLGICs, we studied the nACh $\alpha 4\beta 2$ receptor (Hales *et al.* 2006), which has been well characterised at the single channel level (e.g. Nelson *et al.* 2003). Figure 2 gives a sequence alignment of relevant segments of 5-HT₃ and nACh receptor subunits and highlights residues homologous to R432, R436 and R440 of the human 5-HT_{3A} receptor. In the rat nicotinic $\alpha 4$ subunit these are E584, F588 and E592 and in the rat $\beta 2$ subunit E439, Q443 and E447, respectively. Notably, these residues are either neutral or opposite in sign to those of the 5-HT_{3A} receptor. Co-expression of mutant $\alpha 4$ E584R, or F588R, subunits with wild-type $\beta 2$ subunits yielded functional channels with γ values that were modestly, but significantly, reduced relative to wild-type $\alpha 4\beta 2$ receptors. In the case of the mutant $\beta 2$ E439R subunit co-expressed with wild-type $\alpha 4$, γ was effectively halved, whereas similarly expressed $\beta 2$ Q443R demonstrated only a small decrement in γ . As anticipated, when mutations were simultaneously present in both $\alpha 4$ and $\beta 2$ subunits (i.e. $\alpha 4$ E584R/ $\beta 2$ E439R or $\alpha 4$ F588R/ $\beta 2$ Q443R), γ was markedly reduced. Unfortunately, the influence of the mutations $\alpha 4$ E592R and $\beta 2$ E447R could not be evaluated due to limited functional expression (Hales *et al.* 2006). Collectively, such data indicate that the γ of a representative nACh receptor is, like the 5-HT_{3A} receptor, influenced by select residues within the MA stretch.

The role of the MA stretch in anion-selective pLGICs

As noted by Unwin (2005), the net charge over the MA stretch of the majority subunits of cation-selective pLGICs is negative whereas for anion-selective subunits it is positive. This suggests that the MA stretch of GABA_A and glycine receptors may also influence γ . For the human homomeric $\alpha 1$ glycine receptor there is direct experimental evidence to support this since Carland *et al.* (2009) found that charge reversal (i.e. lysine to glutamate mutations) performed collectively at four locations within the MA stretch reduced inwardly directed γ from approximately 92 to 60 pS. However, outwardly directed single channel currents were largely unaffected by such mutations and selectivity between monovalent anions and cations was unperturbed (Carland *et al.* 2009).

The influence of the MA stretch in GABA_A receptors has not been addressed experimentally (but see Mokrab *et al.* 2007 for a theoretical treatment), other than in a very recent study that reported the application of a synthetic peptide mimicking the MA stretch helix of the GABA_A receptor $\gamma 2$ subunit to inside-out patches excised from hippocampal neurones to reduce γ . Such an effect was interpreted to result from reduced receptor clustering mediated by self-association between MA stretch elements of adjacent GABA_A receptors (Everitt *et al.* 2009).

The influence of the ECD upon γ

Amino acid residues within the ECD may also strongly influence γ and ion selectivity. The extracellular vestibule of the nAChR of *Torpedo marmorata* is approximately 20 Å wide and is abundant in glutamate and aspartate residues that are distributed throughout its 60 Å length creating a strongly electronegative environment that locally concentrates cations (Unwin, 2005; Corry, 2006; Song & Corry, 2009). By contrast, positive charges predominate over negative in the lining of the extracellular vestibule of the $(\alpha 1)_2(\beta 2)_2\gamma 2$ GABA_A receptor (O'Mara *et al.* 2005). The theoretical effects of an electronegative ECD in cation-selective channels were considered over 20 years ago (Dani, 1986; Jordan, 1987) and can be summarised as: (i) an elevation of cation concentration within the vestibule relative to bulk solution and enhanced γ , particularly at low electrolyte concentration; (ii) selection for cations over anions and divalent cations over monovalent cations, and (iii) binding (stabilization) of cations within the vestibule.

Molecular dynamics (MD) simulations of ion transport performed upon a homology model of the adult nAChR of the neuromuscular junction (i.e. $(\alpha 1)_2\beta 1\delta\epsilon$) suggest that rings of charged and polar residues within the ECD participate in determining γ and ion selectivity. Specifically, 16 ns duration MD simulations of Na⁺ ions in flux reveal them to be stabilised at positions corresponding to N47, E83 and D97 of the human $\alpha 1$ subunit sequence (Wang *et al.* 2008). The importance of the nACh $\alpha 1$ D97 and the homologous 5-HT_{3A} D127 subunit residue has recently been proven experimentally. Mutation of the nACh $\alpha 1$ D97 residue and homologous residues of the $\beta 1$, δ and ϵ subunits to lysine produced graded decreases in γ up to a maximum of approximately 80% when all five subunits were mutated (Hansen *et al.* 2008). We found that mere neutralisation of the 5-HT_{3A} (QDA) receptor D127 residue by mutation to asparagine reduced γ from approximately 41 to 8 pS. Moreover, relative permeability to Ca²⁺ was greatly depressed (P_{Ca}/P_{Cs} reduced from 3.55 to 0.12) (Livesey *et al.* 2009). Clearly, the ECD exerts an important control over not only γ , but selectivity between mono- and divalent cations also.

Concluding remarks

Data obtained over the last six years from cation-selective pLGICs strongly support the concept of an extended ion permeation pathway within pLGICs where the ICD and ECD participate with the TM2 domain in determining γ and ion selectivity. Recent findings suggest that a similar scenario might pertain in the anion selective pLGICs. Clearly, there is much scope for further experimentation in this field of fundamental research.

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