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# A review of experimental techniques used for the heterologous expression of nicotinic acetylcholine receptors

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5 expression of nicotinic acetylcholine receptors  
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54 Invited review: Special issue of *Biochemical Pharmacology*  
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59 **Abstract**  
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Nicotinic acetylcholine receptors (nAChRs) are members of the Cys-loop family of neurotransmitter-gated ion channels, a family that also includes receptors for  $\gamma$ -aminobutyric acid, glycine and 5-hydroxytryptamine. In humans, nAChRs have been implicated in several neurological and psychiatric disorders and are major targets for pharmaceutical drug discovery. In addition, nAChRs are important targets for neuroactive pesticides in insects and in other invertebrates. Historically, nAChRs have been one of the most intensively studied families of neurotransmitter receptors. They were the first neurotransmitter receptors to be biochemically purified and the first to be characterized by molecular cloning and heterologous expression. Although much has been learnt from studies of native nAChRs, the expression of recombinant nAChRs has provided dramatic advances in the characterization of these important receptors. This review will provide a brief history of the characterization of nAChRs by heterologous expression. It will focus, in particular, upon studies of recombinant nAChRs, work that has been conducted by many hundreds of scientists during a period of almost 30 years since the molecular cloning of nAChR subunits in the early 1980's.

**Keywords:**

Nicotinic acetylcholine receptor

Heterologous expression

## 1. Introduction

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5 Nicotinic acetylcholine receptors (nAChRs) are neurotransmitter-gated ion channels  
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7 containing five polypeptide subunits that are arranged around a central transmembrane  
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9 pore [1]. As has been reviewed elsewhere, nAChRs have been implicated in several  
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11 human neurological and psychiatric disorders [2-4] and are also an important target site  
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13 for insecticides [5, 6]. In both vertebrate and invertebrate species, nAChRs form a  
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15 diverse family of receptors assembled from a wide range of subunit combinations [6, 7].  
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17 For example, 17 different nAChR subunits ( $\alpha$ 1- $\alpha$ 10,  $\beta$ 1- $\beta$ 4,  $\gamma$ ,  $\delta$  and  $\epsilon$ ) have been  
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19 identified in higher vertebrate species and are known to assemble into a diverse family  
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21 of receptors with distinct subunit compositions [7]. Invertebrate species express a  
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23 similarly diverse population of nAChRs, although less is known about their subunit  
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25 composition [6, 8].  
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34 Whilst studies conducted with endogenously expressed nAChRs continue to provide  
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36 invaluable information, the impact of studies performed with heterologously expressed  
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38 nAChRs has been enormous. As will be discussed in this review, such studies have led to  
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40 dramatic advances in our understanding of the structural, pharmacological and  
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42 biophysical properties of nAChRs. In addition, studies with recombinant nAChRs have  
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44 had clear practical benefits, for example in the identification of a large number of  
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46 subtype-selective small molecules (agonists, antagonists and allosteric potentiators),  
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48 some of which have great potential as either research tools or as lead compounds in  
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50 therapeutic drug discovery [9].  
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1 For many years, nAChRs were the best characterized of any neurotransmitter receptors.  
2 To a large extent, this was due to the availability of a highly abundant source of the  
3 receptor: the electric organ of fish such as the freshwater eel *Electrophorus* (the 'electric  
4 eel') and the marine ray *Torpedo* (reviewed in [10]). As a consequence, the electric  
5 organ nAChR, was the first nAChR to be biochemically purified and the first to be cloned  
6 and expressed. The electric organ has also provided an excellent source of material for  
7 studies aimed at elucidating the structure of the nAChR. Over a period of several years,  
8 the three-dimensional structure of the electric organ nAChR has been revealed at  
9 increasingly high resolution (most recently at a resolution of 4Å) by electron microscopy  
10 [11]. As a result of these studies, the nAChR remains one of relatively few  
11 transmembrane proteins for which high-resolution three-dimensional structural data is  
12 available.

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32 In mammals and other higher vertebrates, nAChRs are expressed at the neuromuscular  
33 junction ('muscle-type' nAChRs) and also in the nervous system, for example in the  
34 brain and autonomic ganglia ('neuronal' nAChRs). In terms of subunit composition and  
35 pharmacological properties, the electric organ nAChR is most closely related to muscle-  
36 type nAChR. Vertebrate muscle-type nAChRs have a subunit composition of either  
37  $(\alpha 1)_2\beta 1\gamma\delta$  or  $(\alpha 1)_2\beta 1\delta\varepsilon$  in fetal and adult muscle, respectively, whereas vertebrate  
38 neuronal nAChRs comprise a heterogeneous population of receptors of diverse subunit  
39 composition, assembled from  $\alpha 2$ - $\alpha 10$  and  $\beta 2$ - $\beta 4$  subunits [7] (note; the  $\alpha 8$  subunit has  
40 been identified in avian but not in mammalian species).

## 51 52 53 54 55 56 57 **2. Early (pre-molecular cloning) expression studies**

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Prior to the molecular cloning of nAChRs, functional reconstitution of the receptor was achieved by the introduction of purified electric organ nAChRs into both lipid vesicles [12-14] and planar lipid bilayers [15, 16]. Such techniques have been used subsequently to characterize nAChRs purified from a variety of sources including chick optic lobe [17, 18], cerebellum [19] and insect tissue [20, 21]. Other expression studies conducted prior to the molecular cloning of nAChRs have used mRNA purified from *Torpedo* electric organ. This has included *in vitro* translation, both in cell-free systems [22-24] and in the presence of cell microsomes (to permit protein glycosylation) [24]. In addition, mRNA purified from *Torpedo* electric organ has been expressed successfully by injection into *Xenopus* oocytes [23, 25]. Importantly, these early oocyte expression studies provided evidence that injection of heterologous mRNA enabled the expression of functional nAChRs that could be activated by acetylcholine [25]. Subsequently, further studies have been conducted in oocytes using mRNA isolated from a variety of other species and tissues. These include studies with mRNA preparations isolated from vertebrate tissues [26-28], cultured cell lines [29] and from invertebrate species [30-33].

Another technique developed for expression of nAChRs in *Xenopus* oocytes, which does not require molecular cloning of the gene of interest, involves the transplantation of membranes from other cells or tissues. This has been applied successfully to membranes isolated from *Torpedo* electric organ [34] and from cultured mammalian cells [35].

### 3. Molecular cloning of nAChRs

Over a two-year period in the early 1980's (1982-1983), several papers were published that described the isolation of cDNAs encoding the four subunits of the *Torpedo* electric

1 organ nAChR (the  $\alpha$  [36-38],  $\beta$  [39],  $\gamma$  [40, 41] and  $\delta$  [39] subunits). Following the  
2 cloning of *Torpedo* nAChRs, nAChR subunit cDNAs have been cloned from numerous  
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4 other species, including the vertebrate muscle-type nAChR subunits ( $\alpha 1$ ,  $\beta 1$ ,  $\gamma$ ,  $\delta$  and  $\epsilon$ )  
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6 [42-47] and the vertebrate neuronal subunits ( $\alpha 2$ - $\alpha 10$  and  $\beta 2$ - $\beta 4$ ) [48-58]. In addition,  
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8 nAChRs have been cloned from numerous invertebrate species [6, 59-61]. For example,  
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10 ten nAChR subunits (D $\alpha 1$ -D $\alpha 7$  and D $\beta 1$ -D $\beta 3$ ) have been cloned from the model insect  
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12 species *Drosophila melanogaster* [62-70]. In general there is relatively high conservation  
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14 of amino acid sequence between nAChR subunits from different species. For example,  
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16 some insect nAChR subunits share 30-45% amino acid sequence identity to their closest  
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18 human homolog, whereas some other subunits have very much greater sequence  
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20 diversity [71].  
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#### 30 **4. Expression of recombinant nAChRs *in vitro* and in bacteria and yeast cells**

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32 In addition to *in vitro* translation studies using tissue-purified mRNAs, *in vitro*  
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34 translation has also been used to express cDNAs encoding nAChR subunits, for example  
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36 to examine subunit transmembrane topology [72]. Bacteria have also been used to  
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38 express recombinant nAChR subunits, despite bacterial cells lacking the machinery for  
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40 appropriate post-translational processing. Indeed, specific binding of nicotinic  
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42 radioligands such as [<sup>125</sup>I]- $\alpha$ -bungarotoxin has been reported with bacterial-expressed  
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44 subunit proteins [55, 73, 74]. Yeast cells have also been used as an expression system for  
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46 nAChRs and have been shown to produce subunit proteins with molecular weights  
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48 similar to those of native nAChRs, suggesting that nAChR subunits expressed in yeast  
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50 undergo glycosylation and signal-sequence cleavage [75-77]. More recently, purification  
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52 and crystallization of the N-terminal domain of the mouse nAChR  $\alpha 1$  subunit expressed  
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in yeast has enabled its three-dimensional structure to be determined at atomic resolution [78].

## 5. Expression of recombinant nAChRs in *Xenopus* oocytes

Just as the *Torpedo* nAChR was the first neurotransmitter receptor to be expressed in *Xenopus* oocytes from tissue-purified mRNA (as discussed above), it was also the first recombinant neurotransmitter receptor to be expressed in oocytes. In 1984, the successful functional expression of a recombinant nAChR was reported, using cDNAs encoding the *Torpedo*  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$  subunits [79]. This was achieved by a somewhat indirect route: cultured mammalian (COS) cells were first transfected with nAChR subunit cDNA constructs, after which the transcribed cRNAs were then isolated from COS cells and microinjected into *Xenopus* oocytes. In later studies, functional expression was achieved in *Xenopus* oocytes using cRNA that had been transcribed *in vitro* from nAChR subunit cDNAs, using purified RNA polymerase [80]. In addition, a more direct approach of injecting cDNA directly into the oocyte nucleus has also been used successfully for the functional expression of nAChRs, first being used to characterize the neuronal  $\alpha 4\beta 2$  nAChR [81]. An approach that has been used extensively to increase expression levels of nAChRs (and of other recombinant proteins) in *Xenopus* oocytes is to replace the 5' untranslated region (UTR) of the cDNA/cRNA with the corresponding UTR of a gene such as *Xenopus*  $\beta$ -globin [82]. There is also evidence that the presence or absence of 5' UTRs can influence the stoichiometry of oocyte-expressed nAChRs such as  $\alpha 4\beta 2$  [83].

Following these early studies, heterologous expression in *Xenopus* oocytes is now used extensively to characterize recombinant nAChRs and has been used very productively

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for numerous combinations of vertebrate nAChR subunits, including  $\alpha 1\beta 1\gamma\delta$  [84],  $\alpha 1\beta 1\delta\epsilon$  [84],  $\alpha 2\beta 2$  [50, 51],  $\alpha 2\beta 4$  [85],  $\alpha 3\beta 2$  [86],  $\alpha 3\beta 4$  [85],  $\alpha 4\beta 2$  [86],  $\alpha 4\beta 4$  [85],  $\alpha 6\beta 4$  [87],  $\alpha 7$  [55],  $\alpha 8$  [18, 88],  $\alpha 9$  [57] (for a more extensive list of recombinant and native nAChR subtypes, see [7]). In addition, *Xenopus* oocytes have been used successfully to examine nAChR subunits cloned from several invertebrate species, including the aphid *Myzus persicae* [89], the brown planthopper *Nilaparvata lugens* [90], the fruit fly *Drosophila melanogaster* [69, 91], the locust *Schistocerca gregaria* [92] and the nematode *Caenorhabditis elegans* [93, 94]. Frustratingly, the heterologous expression of invertebrate nAChRs has proved to be extremely difficult [59, 95] and, in several instances, this has often been achieved only by co-expression with vertebrate nAChR subunits [65, 91, 96, 97]. More recently, expression studies in *Xenopus* oocytes have identified a family of anion-selective nAChRs, cloned from both the nematode *Caenorhabditis elegans* [98] and the mollusc *Lymnaea stagnalis* [99, 100].

Although there is evidence to suggest that, in some cases, ion channel properties of nAChRs expressed in oocytes may differ from those of expressed in mammalian cells [29, 101, 102], *Xenopus* oocytes have proved to be an extremely useful tool for the heterologous expression of nAChRs. In addition, despite oocyte expression being a single-cell technique, methods have been developed in recent years to permit higher-throughput screening of ion channels, such as the nAChRs, expressed in *Xenopus* oocytes. This has been possible due to the development of automated systems for both oocyte injection and for electrophysiological recording [103, 104].

## 6. Expression of recombinant nAChRs in cultured cell lines

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Although functional expression of recombinant nAChRs in *Xenopus* oocytes was first demonstrated in 1984 [79], it was not until 1987 that successful functional expression of nAChRs was achieved in a cultured mammalian cell line [105]. As with the early expression studies in *Xenopus* oocytes, the first functional expression of a nAChR a cultured cell line was of the electric organ nAChR from *Torpedo* [105]. However, following on from this success, there have been numerous heterologous expression studies in cultured cell lines with vertebrate muscle and neuronal nAChR subunit combinations, for example  $\alpha 1\beta 1\gamma\delta$  [106, 107],  $\alpha 1\beta 1\delta\epsilon$  [107],  $\alpha 2\beta 2$  [108],  $\alpha 3\beta 2$  [109],  $\alpha 3\beta 4$  [110],  $\alpha 4\beta 2$  [111],  $\alpha 4\beta 4$  [109],  $\alpha 6\beta 2$  [112],  $\alpha 6\beta 4$  [112],  $\alpha 7$  [113],  $\alpha 8$  [109] (for a more extensive list of recombinant and native nAChR subtypes, see [7]). In many cases these studies have relied upon expression of nAChR subunits cloned downstream from constitutive viral promoters but several inducible promoters have also been used successfully for the expression of nAChRs, including those induced with sodium butyrate [105], dexamethasone [111], tetracycline [114] and heavy metals [96].

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Interestingly, the functional expression of *Torpedo* nAChRs in mammalian cells requires incubation of the transfected cells at temperatures lower than 37°C (e.g. 20-28°C) [105, 115]. The shift to a lower temperature is necessary to allow appropriate folding of *Torpedo* nAChR subunit proteins prior to receptor assembly [115], presumably a consequence of nAChRs from species such as *Torpedo* being adapted to fold efficiently at temperatures lower than 37°C. Similarly, insect nAChR subunits, when expressed in mammalian cell lines, also require incubation at a temperature lower than 37°C for efficient subunit folding and assembly [96, 116, 117]. To an extent, these problems with insect nAChRs can be avoided by expression in insect cell lines [62, 96, 116, 118], which are typically maintained at 20-25°C (although difficulties remain with the expression of

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some invertebrate nAChRs, as will be discussed in more detail below). In addition, although mammalian nAChRs generally fold efficiently in cells maintained at 37°C (as would be expected), there have been reports of enhanced levels of folding, assembly and functional expression of mammalian nAChRs in cells cultured at temperatures lower than 37°C [119-121].

More recently, the availability of cell lines expressing recombinant nAChRs, combined with the use of calcium-sensitive fluorescent dyes, has enabled the development of high-throughput screening assays that are now widely used in drug discovery applications [122-124].

## 7. Expression of recombinant nAChRs in whole animal models

In addition to studies conducted in expression systems such as cultured cells and the *Xenopus* oocytes (discussed above), a variety of techniques have been developed that enable recombinant nAChRs to be examined in whole animal models. In addition to studies in mice (which will be discussed in more detail below), expression studies have also been conducted in invertebrate animal models such as *Caenorhabditis elegans* [125, 126] and *Drosophila melanogaster* [127], for example to examine receptor distribution [125, 126] or to rescue mutant receptor phenotypes [127].

In addition to the construction of knockout mice in which the expression of individual nAChR subunits has been disrupted (reviewed in [128, 129]), several studies have exploited transgenic knockin techniques as a means to studying the expression of recombinant nAChRs in a whole animal model. Several nAChR gain-of-function and disease-associated mutations have been examined by knockin approaches in mice.

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Knockin mouse models that contain a gain-of-function mutation in the 9' position of the M2 domain include those for nAChR subunits  $\alpha 4$  [130, 131],  $\alpha 6$  [132],  $\alpha 7$  [133] and  $\alpha 9$  [134]. Transgenic approaches have also been used to study mutations associated with congenital myasthenic syndromes (in the  $\alpha 1$ ,  $\delta$  and  $\epsilon$  subunit) [135, 136] and mutations associated with nocturnal frontal lobe epilepsy (in the  $\alpha 4$  subunit) [137-139]. A particularly powerful approach is the targeted re-expression of nAChR subunits in knockout mice by viral-based gene delivery [140], a technique that has been used successfully to examine neuronal nAChR  $\alpha 4$  [141],  $\alpha 6$  [141],  $\alpha 7$  [142], and  $\beta 2$  [143] subunits.

## 8. The influence of subunit composition

The ability to control subunit composition in heterologous expression studies (by the selection of subunit cDNAs or cRNAs) has helped to establish the influence of subunit composition upon ligand-binding and functional properties of nAChRs. For example, early studies with muscle-type nAChRs containing either the  $\gamma$  or  $\epsilon$  subunits helped to explain differences between the ion channel properties of receptors found in embryonic and adult muscle [84]. Similar approaches with neuronal nAChRs have helped to demonstrate that both  $\alpha$  and non- $\alpha$  subunits can influence ligand-binding and ion channel properties [144-146].

Studies with hybrid nAChRs (i.e. recombinant receptors containing subunits co-assembled from two or more different species) have helped to establish the contribution of individual subunits to receptor properties [115, 147-149]. Hybrid nAChRs that contain both insect and vertebrate nAChR subunits have also been used extensively in an attempt to circumvent problems encountered with the inefficient heterologous

1 expression of insect nAChRs [59, 95]. This approach has been adopted for studies of  
2 nAChRs cloned from insect species, including the aphid *Myzus persicae* [150, 151], the  
3 brown planthopper *Nilaparvata lugens* [90, 97], the cat flea *Ctenocephalides felis* [152]  
4 and the fruit fly *Drosophila melanogaster* [91, 96, 118, 153].  
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12 Expression studies with partial combinations of nAChR subunits (for example  
13 containing fewer than the four subunits required to form a fully-assembled pentameric  
14  $(\alpha 1)_2\beta 1\gamma\delta$  muscle-type nAChR) have been used to investigate the influence of subunit  
15 composition upon pharmacological and functional expression [154-159]. Similarly,  
16 studies conducted with partial subunit combinations have been used to investigate the  
17 order of nAChR subunit assembly and to identify possible assembly intermediates [160-  
18 164]. In addition, altering subunit cDNA or cRNA ratios for the heterologous expression  
19 of neuronal nAChRs such as  $\alpha 4\beta 2$  has provided evidence that changes in subunit ratios  
20 can influence subunit stoichiometry [165, 166]. This has provided evidence indicating  
21 that receptors with alternative subunit stoichiometries, for example  $(\alpha 4)_2(\beta 2)_3$  and  
22  $(\alpha 4)_3(\beta 2)_2$  nAChRs, can have significant differences in their agonist sensitivities.  
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## 42 **9. Expression of nAChR subunit chimeras**

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44 A paper published in 1986 described a series of artificial subunit chimeras combining  
45 regions of the *Torpedo* and bovine  $\delta$  subunits [167]. This study, which identified the  
46 importance of the M2 transmembrane domain in determining ion permeation, was the  
47 forerunner of many subsequent studies that have employed subunit chimeras to  
48 examine the properties of nAChRs. A selection of these studies is discussed below.  
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Despite the successful functional expression of the nAChR  $\alpha 7$  subunit in *Xenopus* oocytes [55], considerable problems have been encountered in its efficient expression in some cultured cell lines (reviewed in [95]). An imaginative strategy to circumvent this problem has been the construction of an artificial chimera comprising the N-terminal domain of the  $\alpha 7$  subunit fused to the transmembrane and C-terminal region of the 5-HT<sub>3A</sub> subunit [168]. This  $\alpha 7/5$ -HT<sub>3A</sub> subunit chimera generates a functional ion channel in cultured cell lines that fail to express  $\alpha 7$  efficiently [168]. The construction of nAChR chimeras containing the C-terminal region of the 5-HT<sub>3A</sub> subunit has proved to be a powerful experimental technique and one that has been used subsequently with several other nAChR subunits including  $\alpha 1$  [169],  $\alpha 4$  [119],  $\alpha 8$  [170],  $\alpha 9$  [171],  $\alpha 10$  [171] and  $\beta 2$  [119], as well as with insect nAChR subunits [116]. Other studies have exploited chimeras containing domains from two different nAChR subunits to overcome inefficient functional expression of, for example, the  $\alpha 6$  subunit [172-174].

Construction of a more extensive series of  $\alpha 7/5$ -HT<sub>3A</sub> subunit chimeras has helped to identify subunit domains that are responsible for influencing folding of the  $\alpha 7$  subunit in non-neuronal cell lines [175, 176]. Subunit chimeras have also helped to identify domains that are important in the folding and assembly of muscle-type [163, 177-181] and neuronal [182, 183] nAChRs and to identify domains involved in receptor targeting and trafficking [184-187]. Other studies involving the use of subunit chimeras have helped to investigate receptor properties such as agonist sensitivity [188-195], antagonist sensitivity [190, 192, 196-198], modulation by allosteric modulators [199, 200], desensitization [188], inactivation [201] and channel gating [202-204].

1 Fusion proteins, in which nAChR subunits are linked to proteins such as GFP (green  
2 fluorescent protein) have been useful in detecting recombinant subunits expressed in  
3  
4 either cells or tissues [205-208]. In addition, by using fluorescence resonance energy  
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6 transfer (FRET) methods it has been possible to examine assembly, trafficking and  
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8 subunit stoichiometry of nAChRs in cultured neuronal cells [209, 210].  
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15 Expression of nAChR subunits in which selected domains have been deleted is a further  
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17 approach that has been used in a number of heterologous expression studies aimed at  
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19 identifying regions involved in assembly [211-213], subunit topology [72] and cell-  
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21 surface expression [214, 215].  
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## 26 27 **10. Expression of nAChRs altered by site-directed mutagenesis**

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29 In 1985, the use of site-directed mutagenesis combined with heterologous expression  
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31 enabled the identification of regions and individual amino acids within the *Torpedo*  $\alpha$   
32  
33 subunit that influence ligand binding and functional expression [80]. This was the first  
34  
35 of many hundreds of studies that have employed site-directed mutagenesis to  
36  
37 characterize nAChRs. Other such early studies employing site-directed mutagenesis  
38  
39 were aimed at identifying amino acids influencing ion channel properties [216, 217]. It  
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41 would be impractical to attempt to provide a comprehensive review of all mutagenesis  
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43 studies conducted with nAChRs. Nevertheless, some examples are discussed below.  
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52 Mutations at the 9' position within the nAChR subunit M2 domain (such as the L247T  
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54 mutation in the  $\alpha 7$  subunit [218]) have particularly dramatic effects. Mutations at this  
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56 position in  $\alpha 7$  alter receptor desensitization, rectification, agonist potency, and  
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58 antagonist effects [218-222]. Similarly complex effects have been reported for  
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1 mutations at other positions within the M2 domain of  $\alpha 7$  (for example, the 6' position  
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7 An exhaustive list of nAChR amino acids examined by site-directed mutagenesis would  
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9 be prohibitively long. Site-directed mutagenesis, in combination with heterologous  
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11 expression, has however been used successfully to examine phenomena such as, subunit  
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13 glycosylation [225-227], the role of disulfide-linked cysteines [80, 228], cell surface  
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15 receptor trafficking [214, 229, 230], interactions with agonists and antagonists [231-  
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17 233], modulation by zinc [234, 235] and by other allosteric modulators [199, 200],  
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19 calcium permeability [236] and channel gating [202, 237-241]. Analysis of mutated  
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21 nAChRs has also provided insights into how subunit domains may move during receptor  
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23 activation [241, 242]. In addition, analysis of double mutations by mutant cycle analysis  
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25 is a powerful approach by which to investigate protein interactions, such as those  
26  
27 between nAChR subunits and peptide ligands (see, for example, [243, 244]). Another  
28  
29 dramatic example of the application of site-directed mutagenesis is illustrated by the  
30  
31 ability to convert the  $\alpha 7$  nAChR into an anion-selective channel [245, 246]. Reporter  
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33 mutations, introduced by site-directed mutagenesis, have been used to examine subunit  
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35 stoichiometry of heteromeric nAChRs [247-250]. Heterologous expression studies have  
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37 also helped to identify the consequences of naturally occurring nAChR mutations  
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39 associated with human disorders such as congenital myasthenic syndrome and epilepsy  
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41 (see, for example [2, 4, 251, 252]).  
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54 Cysteine-scanning mutagenesis, combined with cysteine-reactive compounds, has been  
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56 a powerful and extensively used technique to examine nAChRs [253-255]. Another  
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58 powerful experimental approach is the incorporation of unnatural amino acids into  
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1 recombinant nAChRs. This has been achieved by means of site-directed mutagenesis  
2 combined with nonsense codon suppression (i.e. modified tRNAs containing unnatural  
3 amino acids) [256]. This is an approach that has been used successfully to examine the  
4 role of amino acids located at the agonist binding site [256, 257] and within the ion  
5 channel pore [258].  
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### 11 **11. Expression of nAChR subunit concatemers**

12 Artificial subunit concatemers (containing two nAChR subunits fused into a single  
13 'tandem' polypeptide) have been used to examine issues such as subunit stoichiometry  
14 [259-261], although there have been reports that, in some cases, their incorporation into  
15 assembled nAChRs may not always occur as might be expected [260, 262]. A more  
16 ambitious recent approach aimed at constraining subunit stoichiometry has been the  
17 generation of five-subunit concatemers [263, 264]. Studies such as these have helped to  
18 confirm that differences in pharmacological properties (such as high and low agonist  
19 sensitivity, as discussed above) can be a consequence of alternative subunit  
20 stoichiometries, for example  $(\alpha 4)_2(\beta 2)_3$  and  $(\alpha 4)_3(\beta 2)_2$ .  
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### 42 **12. Nicotine-induced up-regulation examined with recombinant nAChRs**

43 Chronic exposure to nicotine, as occurs during tobacco smoking causes an upregulation  
44 of nAChRs in the brain [265, 266]. In addition to numerous studies conducted with  
45 native nAChRs, nicotine-induced upregulation has also been examined extensively in  
46 heterologous expression systems, including both *Xenopus* oocytes [267, 268] and  
47 cultured cell lines [267, 269-275]. Such studies have helped to confirm that nicotine-  
48 induced upregulation is a post-transcriptional event and that it may occur by a  
49 mechanism consistent with nicotine acting as a molecular chaperone [276].  
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### 13. Co-expression with chaperones and interacting proteins

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5 Studies in transfected cells have helped in characterizing the interaction of nAChRs with  
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7 chaperone proteins such as BiP [277, 278] and calnexin [279-281]. Such studies have  
8  
9 also helped to reveal the role of nAChR-interacting proteins such as 14-3-3 [282, 283]  
10  
11 and VILIP-1 [284] in regulating cell-surface expression of  $\alpha 4\beta 2$  nAChRs. Co-expression  
12  
13 studies of muscle nAChRs with the cytoplasmic receptor-associated protein rapsyn (also  
14  
15 referred to as '43K protein') have helped to establish the role of rapsyn in nAChR  
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17 clustering by means of expression studies in both *Xenopus* oocytes [285] and in  
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19 transfected cell lines [286].  
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27 More recently, the role of an ER-resident transmembrane chaperone protein, RIC-3, has  
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29 been examined and found to exert a dramatic effect on the maturation of several nAChRs  
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31 (reviewed in [287]). RIC-3 was originally cloned from *C. elegans* [288] but has been  
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33 cloned subsequently from both mammalian and insect species [289, 290]. Co-expression  
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35 of RIC-3 in *Xenopus* oocytes or cultured cell lines results in enhanced levels of functional  
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37 expression of several nAChRs subtypes [289, 290], but has a particularly profound effect  
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39 on nAChRs such as  $\alpha 7$ . As has been discussed elsewhere [95, 287], severe difficulties  
40  
41 have been encountered in obtaining functional expression of recombinant  $\alpha 7$  nAChRs in  
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43 several cultured mammalian cell lines [291-296]. Recent studies have revealed that co-  
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45 expression of  $\alpha 7$  with RIC-3 in such non-permissive cells facilitates appropriate folding  
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47 and functional expression of  $\alpha 7$  nAChRs [290, 297, 298]. In addition, it has been  
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49 reported that co-expression of  $\alpha 4\beta 2$  nAChRs with UNCL, a mammalian homologue of the  
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51 *C. elegans* transmembrane protein UNC-50, results in increased nAChR functional  
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53 expression [299], although it has been suggested that this may be due to an RNA-binding  
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1 activity rather than to a chaperone-like effect on the receptor protein. Interestingly, a  
2 recent study has demonstrated that successful functional expression in *Xenopus* oocytes  
3 of a levamisole-sensitive nAChR from *C. elegans* requires the co-expression of five  
4 different nAChR subunits, together with three different chaperone or enhancer proteins  
5 (RIC-3, UNC-50 and UNC-74) [300].  
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#### 11 **14. Conclusion**

12 The aim of this review has been to give a brief overview of the variety of heterologous  
13 expression strategies that have been used to examine nAChRs. In addition, some  
14 examples of how these approaches have provided information concerning the structural  
15 and functional properties of nAChRs have been discussed. Clearly, an enormous amount  
16 has been achieved in the 30-years that have elapsed since the description in 1979 of the  
17 functional reconstitution of nAChRs in lipid vesicles. As a consequence, it is impractical  
18 for a short review such as this to provide a comprehensive account of all nAChR  
19 expression studies. The choice of examples selected for inclusion has, inevitably, been  
20 somewhat subjective as well as being subject to length constraints (300 references).  
21 Nevertheless, it is hoped that this review provides a useful summary of the huge amount  
22 that has been achieved by a large number of scientists over the past three decades.  
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51 provided by Bayer CropScience, Bayer HealthCare, Eli Lilly and Syngenta.  
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#### 59 **Figure Legends**

1  
2 **Figure 1 Nicotinic acetylcholine receptors structure and subunit topology.**  
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5 A) Diagrammatic representation of a nicotinic acetylcholine receptor (nAChR),  
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7 illustrating the pentameric arrangement of subunits arranged around a central cation-  
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9 selective pore. The five subunits traverse the plasma membrane, with the agonist  
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11 binding domain located on the extracellular face of the membrane. B) Three  
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13 dimensional structure of an individual nAChR subunit illustrating the topology of the  
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15 polypeptide backbone. The image is derived from the 4Å resolution structure (Protein  
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17 Data Bank accession number 2BG9) of the *Torpedo* nAChR [11]. Each subunit contains  
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19 an extracellular agonist-binding domain, four  $\alpha$ -helical transmembrane domains and a  
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21 large intracellular domain (located between the third and fourth transmembrane  
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23 domains). This intracellular domain contains the greatest sequence diversity between  
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25 subunits but is not well resolved in the 4Å *Torpedo* nAChR structure and, as a  
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27 consequence, only the short amphipathic  $\alpha$ -helical domain is illustrated.  
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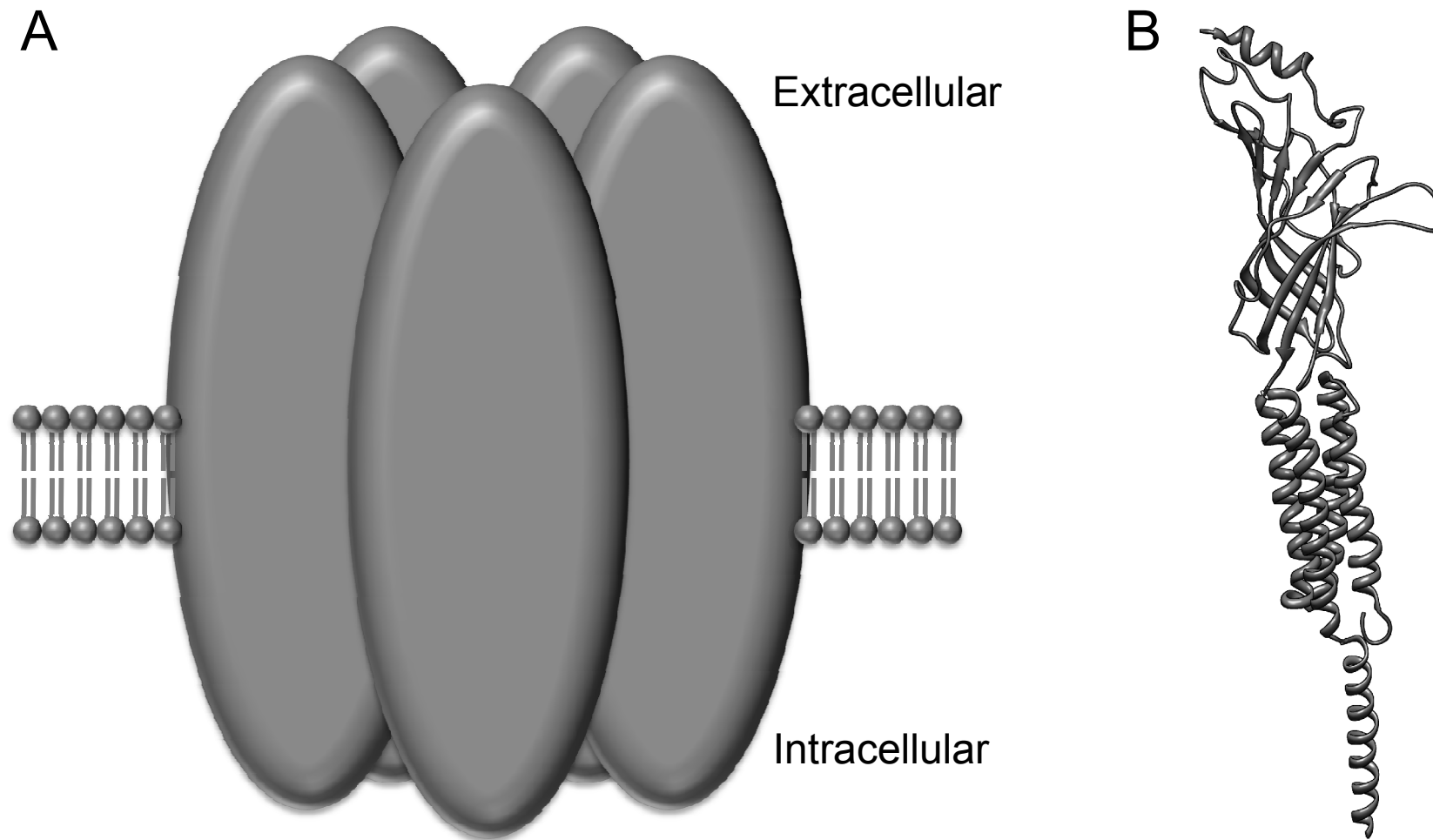
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