Br. J. Pharmacol. (1992), **105**, 657–666

α4-2β2 and other nicotinic acetylcholine receptor subtypes as targets of psychoactive and addictive drugs

[1]John Connolly, Jim Boulter & Stephen F. Heinemann

Molecular Neurobiology Laboratory, The Salk Institute, P.O. Box 85800, San Diego, CA 92138, U.S.A.

1 *Xenopus* oocytes were injected with various muscle and neuronal nicotinic acetylcholine receptor (ACh receptor, cholinoceptor) subunit RNA combinations and their pharmacological properties studied using two-electrode voltage clamp. The functional expression of one of these combinations, rat α4-2β2, has not been previously described. The α4-2 mRNA is a splicing variant transcribed from the α4 gene. In the experiments reported here, the α4-2β2 subtype was functionally indistinguishable from the α4-1β2 subtype.

2 For each subtype, the relative potency of nicotine compared with acetylcholine was obtained by estimating the relative concentration of nicotine which would elicit the same current response as 0.1 μM ACh. The ratios of these concentrations (nicotine: ACh) for the mouse muscle ACh receptor-(α1β1γδ) was 96.1:1. In contrast, the ratios for the rat neuronal subtypes were: α2β2, 1.01:1; α3β2, 2.01:1; α4β2, 0.76:1 and α4-2β2, 0.76:1. The much greater relative nicotine sensitivity of the neuronal subtypes as compared with muscle receptors illustrates their potential to mediate the psychoactive and addictive effects of nicotine. However, it does not appear that the differences in relative nicotinic sensitivity among the neuronal receptors themselves can be used as a simple discriminative tool in neuronal tissue.

3 The slopes of the log dose-log response curves at low ACh concentrations were all greater than 1 but less than 2, suggesting that at least two agonist binding sites mediate the functional response of each hetero-oligomer.

4 The response of all the neuronal subtypes to ACh could be inhibited by the psychoactive drugs mecamylamine, amitriptyline, phencyclidine, trifluoperazine and promethazine. With the exception of the very potent antagonist, mecamylamine, the degree of block of the peak current to ACh produced by 10 μM concentrations of these drugs was remarkably similar (around 50%).

5 The degree of inhibition produced when the antipsychotic drug, trifluoperazine, was co-applied with ACh increased as the duration of application increased. Such an effect was not observed with promethazine, a related phenothiazine derivative which does not have antipsychotic actions.

Keywords: Nicotinic acetylcholine receptors; voltage-clamp; ooctyes; psychoactive; nicotine; drug design; α4-2 cDNA; schizophrenia

Introduction

The psychological actions of nicotine are important reinforcers of the smoking habit (Benowitz, 1988, review). Some of the objectively confirmed effects reported by smokers include arousal, mood-elevation, improved concentration and a reduction in feelings of stress and anxiety (Wesnes & Warburton, 1983; Warburton, 1987). In contrast, the selective loss of cortical, high-affinity, nicotine binding sites from the brain in Alzheimer's disease is correlated with a loss of cognitive function (Whitehouse *et al.*, 1988). Apart from their obvious role in addiction, an additional implication of these observations is that nicotinic acetylcholine receptors (ACh receptors, cholinoceptors) are important elements in some of the neuronal circuits that mediate psychological functions.

In order to obtain a more complete understanding of the pharmacological and molecular bases of these phenomena, we decided to examine the effects of nicotine and other psychoactive drugs on a variety of nicotinic ACh receptor subtypes. cDNAs whch encode subunits of muscle and neuronal nicotinic ACh receptors have been isolated from several species and in many cases their corresponding mRNAs have been functionally expressed (Deneris *et al.*, 1991, review, Couturier *et al.*, 1990a). The cDNAs encode polypeptides which fall into two major classes. A major distinguishing feature of one class, the α-subunits, is that each subunit contains two consecutive cysteines at amino-acid positions 192,193 (*Torpedo* numbering) which are thought to be in the vicinity of the agonist binding site (Kao *et al.*, 1984). The α-subunit in muscle is called α1, and to date those found in neuronal receptors have been numbered in the historical order in which they were discovered, from α2 to α7. There are also ACh receptor subunits which do not contain these two cysteines. Members of this latter class include the mouse muscle β1, γ and δ subunits, and the rat neuronal β2 and β4 subunits. Among several demonstrations of expression, it has been shown that functional nicotinic ACh receptors can be detected following the co-injection of the mouse muscle α1β1γδ RNAs into *Xenopus* oocytes (Yoshii *et al.*, 1987; Connolly, 1989). Co-injection of any one of the rat neuronal α2, α3 or α4-1 RNAs with either β2 (Boulter *et al.*, 1987; Deneris *et al.*, 1988, Wada *et al.*, 1988b) or β4 (Duvoisin *et al.*, 1989) RNA also results in robust and reproducible functional expression of nicotinic ACh receptors.

Although the β4 gene is expressed in PC12 cells (Boulter *et al.*, 1990) and embryonic chick ganglia (Couturier *et al.*, 1990a, non-α3 in chick nomenclature), it shows very limited expression in rat brain, being mainly transcribed in the medial habenula (Duvoisin *et al.*, 1989). In contrast the β2 subunit gene is very widely expressed (Wada *et al.*, 1988a). Therefore the neuronal ACh receptor subtypes examined in this study, which was directed towards the understanding of the potential involvement of nicotinic ACh receptors in a variety of brain processes, combined neuronal α-subunits with the more ubiquitous β2 subunit.

In many brain regions, the distribution of the β2 mRNAs was found to overlap with that of α4 mRNAs (Wada *et al.*, 1988a). However, when hypothalamic cDNA libraries were first screened, two different, but equally abundant, species of α4 clones were found (Goldman *et al.*, 1987). It was thought that the two types of α4 mRNAs from which these cDNA clones were derived may have arisen by alternate splicing of the primary transcript of the α4 gene. The first species, which was full length, was called α4-1 and is the subtype we have

[1] Author for correspondence at: Dept. of Physiology and Pharmacology, University of Strathclyde, Royal College, 204 George Street, Glasgow G1 1XW

used in functional expression studies of nicotinic ACh receptors to date (Boulter et al., 1987; Deneris et al., 1988; Wada et al., 1988b). The amino acid sequence of the α4-1 subunit was deduced from the cDNA clone, and the last two amino acids of the carboxy (C)-terminus were predicted to be alanine and cysteine. The α4-2 cDNA was not full length, and differed from the α4-1 cDNA at its 3' end. The deduced amino acid sequence of this cDNA was one amino acid longer at the C-terminus, and ended in the three amino acids glycine, methionine and isoleucine (GMI). The 3' untranslated region of the clone also differed from that of α4-1. For the rest of the overlapping regions of the cDNAs, the sequences were identical. If, like α4-1, the α4-2 subunit can form functional receptors with β2, then it too may be incorporated into central nicotinic ACh receptors. Therefore, in addition to examining the actions of psychoactive drugs on the ACh receptor subtypes described above, we also describe here the first functional expression of the neuronal α4-2 subunit in conjunction with the β2 subunit.

Methods

Expressible clones

A full length α4-2 cDNA was constructed from partial clones by use of conventional molecular biological techniques. The coding region and 5'-end of α4-2 mRNA is identical to that of α4-1 mRNA with the exception of the differences in the 3'-end mentioned above. The other expressible clones used in this study have all been described previously: α1 (Boulter et al., 1985); γ (Boulter et al., 1986b); β1 and δ (Heinemann et al., 1986); α2 (Wada et al., 1988b); α3 and α4-1 (Boulter et al., 1986a; 1987; Goldman et al., 1987) and β2 (Deneris et al., 1988).

In vitro *synthesis of RNA and injection into oocytes*

Plasmid DNA for each subunit constructed was linearised with restriction enzymes at the 3' end of each clone. Diguanosine triphosphate-capped RNA was then transcribed from these DNA templates by bacteriophage SP6 RNA polymerase (Melton et al., 1984).

Oocytes were prepared for injection as described previously (Boulter et al., 1987). This technique removes the follicular cell layer but leaves the vitelline membrane intact. A total of 3–5 ng of RNA was injected into each oocyte, in a volume of 50 nl of H_2O. The mouse muscle ACh receptor (cholinoceptor) subunits (α, β, γ and δ) were injected into the oocytes in the ratio 1:1:1:1. Neuronal ACh receptor subunit combinations were also injected in a 1:1 ratio.

Drug solutions

The agonists (−)-nicotine hydrogen tartrate and acetylcholine chloride and antagonists, phenycyclidine (PCP) hydrochloride, promethazine hydrochloride, amitriptyline hydrochloride, mecamylamine hydrochloride and trifluoperazine hydrochloride, were all obtained from Sigma Co., St. Louis, U.S.A.

Recording procedures

Two-electrode voltage-clamp recordings of the responses of oocytes to various agonists and antagonists were made with a Dagan 8500 voltage-clamp unit. The voltage micropipettes contained 3 M KCl (resistance 2–4 MΩ) and the current micropipettes were filled with 0.25 M CsCl, 0.25 M CsF, 100 mM EGTA, pH 7.2 (resistance 0.5–1 MΩ). The control solution (in which drugs were also dissolved), contained 115 mM NaCl, 1.8 mM CaCl, 2.5 mM KCl, 10 mM HEPES (pH 7.2) and 1 μM atropine.

Individual oocytes were placed in a groove in the base of a narrow perspex chamber. A system of switches close to the bath inlet allowed either drug or control solutions to be perfused at a rate of approximately 30 ml min^{-1} through a bath volume of 0.5 ml. Under these conditions, the current response reached 90% of its initial peak value within 2–3 s (see Figures 1, 4 and 5). Washing out of the drug took slightly longer, perhaps because some of the drug is trapped between the vitelline and cell membranes. With this rate of perfusion, care must be taken to ensure that the oocytes are stably positioned and do not move about the recording pipettes while the solutions are being changed, as this can give rise to small, artefactual inward currents.

Potential interference by endogenous subunits

Spurious results due to the incorporation of endogenous oocyte ACh receptor subunits into receptors expressed after injection of mouse muscle of rat neuronal subunit RNAs does not seem likely. We have found previously, using voltage recordings, that with the exception of a very weak and inconsistent response from α4-1, rat neuronal α and β subunit RNAs injected singly will not form functional receptors (Boulter et al., 1987; Deneris et al., 1988; Wada et al., 1988b, Duvoisin et al., 1989). Similarly, functional expression has also not been detected for singly injected chick α4 and non-α1 (most homologous to rat β2) subunit RNAs (Bertrand et al., 1990). Thus we have no positive evidence that endogenous *Xenopus* subunits which can combine with the neuronal subunits we have injected are present. Nor have we seen nicotinic responses in oocytes following mouse muscle α1 RNA, water, sham or mouse muscle β1γδ RNA injections (Deneris et al., 1991; Connolly, 1989). Similar results have been obtained with *Torpedo* receptor subunits (Kurosaki et al., 1987). In their experiments, Leutje & Patrick (1991) have also found that injections of mouse muscle β1γδ and rat β2 RNAs do not lead to functional expression. Hence if endogenous, compatible, muscle type subunits were present in oocytes used in these experiments, they were expressed at an undetectably low level.

However, there has been a report of functional expression when only mouse muscle β1γδ subunits were injected, but this current was only just detectable at mM concentrations of ACh (Buller & White, 1990). Also, a low level of endogenous alpha-subunit RNA has been reported in oocytes (Hartmann & Claudio, 1990), but the amount per oocyte is less than 0.5 picograms, 10,000 fold less than the nanogram amount of synthesized RNA injected here. If there is endogenous subunit RNA expression at this level in some of the oocytes we have used, then at the concentrations applied here, the above results suggest that such expression would not make a significant contribution to the current responses we observed.

Collection of data for dose-response curve

Currents greater than 2μA could not be reliably clamped under our recording conditions and currents smaller than 1–2 nA were difficult to measure accurately. The steepness of the dose-response curves meant that it was not possible to record the responses to a wide range of concentrations in an individual cell within these current limits. Therefore, to obtain the data depicted in Figures 2 and 3, several trials were made at a standard concentration of ACh during the course of each experiment. The standard concentrations were 1 μM ACh for the α2β2 and α3β2 combinations and 0.1 μM ACh for the α1β1γδ, α4-1β2 and α4-2β2 combinations, all voltage-clamped at −70 mV. The current produced by this standard dose was defined as 100% and the responses of that oocyte to other concentrations of ACh and all concentrations of nicotine, were expressed as a % of it.

For nicotine and ACh concentrations of 1 μM and below, at least duplicate trials for each agonist were performed in each oocyte at each concentration and the responses averaged. At agonist concentrations greater than 1 μM, the responses were not always duplicated, since full recovery from desensitization could take more than 30 min. At these high agonist concentra-

tions, considerable desensitization may have occurred before the peak response was attained. Unfortunately, the large size of oocytes (>1 mm) and the slowing of solution exchange by the vitelline membrane prohibit rapid and simultaneous perfusion of the entire cell surface. This prevents estimation of fast rates of desensitization in this preparation. Additional problems at high agonist concentrations are the probability of channel block by the agonists and the difficulty of comparison with a convenient standard, non-desensitizing, response. Because of these considerations, the dose-response curves were not extended beyond agonist concentrations of 10 μM.

Estimation of relative potencies and low-concentration slopes

Unlike the muscle receptor dose-response curves at low agonist concentrations, the nicotine and ACh dose-response curves plots are not parallel for the neuronal receptors, possibly because the rates of desensitization of neuronal nicotinic ACh receptors are different for ACh and nicotine. For this reason, the potency ratios are compared at concentrations where the curves are closest to parallel. The relative potency of ACh compared to nicotine was then calculated as the antilog of the difference between the logs of the concentrations of nicotine and ACh that gave a particular current response.

In order to estimate these concentrations for a particular subtype, straight lines were fitted by linear regression to the three or four lowest concentration points of the ACh and nicotine plots. Calculated log (current) values corresponding to either 0.1 μM ACh or 0.316 μM ACh were then obtained from the new lines. These log (current) values were then applied to the calculated nicotine curve to obtain the corresponding nicotine concentrations. The antilog of the difference between the logs of these two agonist concentrations provides the ratio shown in Table 1. The low concentration slopes were calculated from the straight lines fitted by linear regression to the lowest 3 concentration points on the log [ACh]-log (current) plots. It was noticed that some batches of oocytes would express receptors with lower mean slopes than others, but data from several batches were pooled to give the results shown here in Figures 2 and 3. Where appropriate, Student's t tests for paired and independent samples were used to test for significance.

Results

Functional expression of the α4-2β2 combination

Figure 1 shows the current responses of an oocyte injected with the α4-2β2 RNA combination to 0.316 μM applications of ACh and nicotine. The strong expression clearly demonstrates that the combination is functional. The dose-response curves for the α4-1β2 and α4-2β2 combinations shown in Figure 3c and 3d are almost superimposable. Analysis of their responses to ACh and nicotine does not reveal any significant difference, the relative potency being the same for each (Table 1).

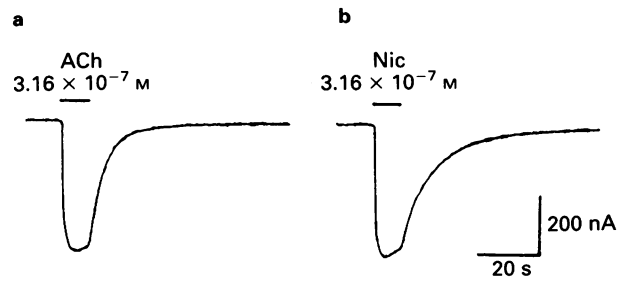

Figure 1 Functional expression in oocytes injected with the α4-2β2 RNA combination. Current responses to 0.316 μM acetylcholine (ACh) (a) and 0.316 μM nicotine (Nic) (b) illustrate typical rates for the application and removal of drug solutions.

Table 1 Summary of characteristics of dose-response plots

Injected	*Relative potency 0.1 μM ACh*	*Relative potency 0.316 μM ACh*	*Slope* (mean ± s.e.)
α1β1γδ	96.1	—	1.71 ± 0.04
α2β2	1.01	1.27	1.22 ± 0.02
α3β2	2.01	2.20	1.49 ± 0.11
α4-1β2	0.76	1.03	1.08 ± 0.12
α4-2β2	0.76	1.02	1.37 ± 0.03

The relative potency indicates the ratio of the concentrations of nicotine and acetylcholine which give the same magnitude of current response. The [nicotine]/[acetylcholine] ratio is shown for two current values, which correspond to the currents elicited by the application of acetylcholine (ACh) at concentrations of 0.1 μM and 0.316 μM. The slope values (± s.e.) are those of lines fitted by linear regression to the lowest three concentration points of the averaged ACh dose-response plots shown in Figures 2 (muscle) and 3 (neuronal). Details of the procedures used to calculate these values are described in the Methods section.

Nicotinic acetylcholine receptor subtypes show differential sensitivity to (−)-nicotine

It has been long appreciated that among native nicotinic ACh receptors, there is a clear differentiation between the relative sensitivity of the muscle and neuronal nicotinic ACh receptors to nicotine, with muscle receptors being much less sensitive to nicotine (Hill, 1909; Curtis & Eccles, 1958; Lukas, 1989). Such differences in the nicotine sensitivities of receptors formed from cloned subunits might also be expected from earlier voltage (Boulter *et al.*, 1987; Duvoisin *et al.*, 1989) and voltage-clamp (Bertrand *et al.*, 1990; Couturier *et al.*, 1990a) studies.

This marked difference is confirmed here with the receptor subtypes we have introduced into *Xenopus* oocytes. The dose-response curves in Figure 2a were obtained from oocytes injected with the RNA combination α1β1γδ. As the summary in Table 1 indicates, it would require 9.6 μM (−)-nicotine to produce the same current response as that produced by 0.1 μM ACh, approximately 2 orders of magnitude difference in the respective agonist concentrations. However, the graphs in Figure 3 demonstrate that the relative sensitivity of the neuronal receptor subtypes to nicotine is much greater. Table 1

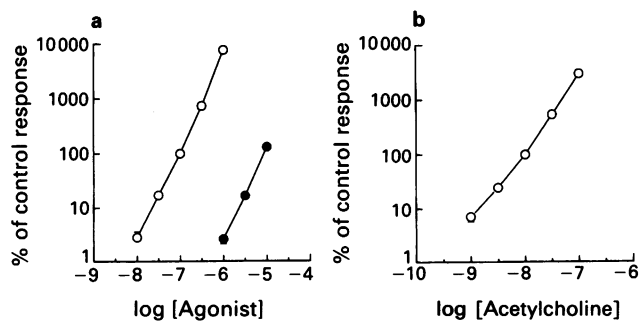

Figure 2 (a) Log-log plots of the agonist dose-current response relationships of acetylcholine (○) and nicotine (●) with oocytes injected with mouse muscle α1β1γ and δ subunit RNAs. Each point represents averaged, normalised (to the 0.1 μM acetylcholine response) data pooled from a minimum of 4 oocytes (see Methods). The current value (± s.e.) corresponding to 100% was 59 ± 12.1 nA (n = 20 oocytes). The oocytes were voltage clamped at −70 mV. Vertical bars, where visible, represent the standard errors at each point. (b) Log-log plot of the acetylcholine dose-current response relationship of oocytes injected with the α4-1β2 combination and voltage clamped at −100 mV. Responses were normalised to the response to 0.01 μM acetylcholine and each point represents the data collected from at least 4 cells. The current value corresponding to the 100% response was 50.1 ± 2.1 nA (n = 5 oocytes). The maximum slope, fitted by linear regression to the points included in the range 10 nM to 100 nM, was 1.47 (± 0.02, s.e.).

shows that the nicotine/ACh potency ratio for neuronal receptors is, at most, approximately 2:1. Even more remarkable is that for the $\alpha 4\beta 2$ combinations, at low concentrations, nicotine is more potent than ACh, the natural transmitter. Thus, when compared with their sensitivity to ACh, neuronal receptors are at least 50 times more sensitive to nicotine than muscle ACh receptors.

Although muscle and neuronal receptors are easily distinguished on the basis of their relative sensitivity, the differences among the neuronal receptors themselves are more subtle. In many of the experiments upon which Figure 2 is based, an identical concentration of both ACh and nicotine was applied to the same oocyte. To test if these more subtle differences were significant, the current response of an oocyte to a given concentration of nicotine was expressed as a % of this response to the same concentration of ACh. The mean of the % nicotine responses from several oocytes was obtained. This was done for those concentrations (0.1, 0.32 and 1 μM) which were common to the neuronal dose-response curves (see Table 2). At all three agonist concentrations, the means of the nicotine responses of the $\alpha 4$-$1\beta 2$ and $\alpha 4$-$2\beta 2$ subtypes were not significantly different from each other. In contrast, the responses of these two combinations were significantly different from the less sensitive $\alpha 2\beta 2$ subtype ($P < 0.05$) and even more easily distinguished from the $\alpha 3\beta 2$ combination ($P < 0.002$). The means of the % nicotine responses of the $\alpha 3\beta 2$ and $\alpha 2\beta 2$ combinations were also different from each other ($P < 0.02$). Thus there are agonist concentrations at which these particular subtypes can be distinguished upon the basis of their relative nicotine sensitivity.

Table 1 also shows the value of the slope of the log dose–log mean current response curve for the muscle receptor at low ACh concentrations. At these low concentrations the slope value will approximate to the Hill coefficient. The value of 1.71 suggests that there are at least 2 agonist binding sites in each mouse muscle receptor hetero-oligomer that we have expressed. The values of the slopes of lines fitted through the lowest three concentration points of each of the neuronal ACh receptor dose-response plots shown in Figure 3 are also listed in Table 1. However the slopes of the neuronal dose-response plots were all lower than that of the muscle ACh receptors (Figure 3, Table 1). The lowest value was obtained for the $\alpha 4$-$1\beta 2$ combination. This combination was re-examined, and to minimize desensitization and increase the sensitivity of measurement of the smaller currents, nanomolar concentrations of acetylcholine and a holding potential of -100 mV were used (Figure 2b). The maximum slope obtained, between 10 nM and 100 nM ACh, was still only 1.47 ± 0.02 ($n = 4$ oocytes). A higher value of 1.83 has been reported for the chick $\alpha 4$ non-$\alpha 1$ combination (Ballivet *et al.*, 1988). The reasons for the low slope values of rat neuronal ACh receptor dose-response curves are not clear, but it may be a consequence of very rapid desensitization, even at these low agonist concentrations. Recently, Cooper and colleagues (1991) have deduced that for the chick $\alpha 4$ non-$\alpha 1$ combination, the stoichiometry is two alpha subunits and three non-α subunits. It would therefore seem most probable that our receptors also contain two α-subunits and three β subunits.

Table 2 Variation in the relative nicotine responses for different neuronal ACh receptor subtypes

	0.1 μM (% ± s.e.)	n	*0.32 μM* (% ± s.e.)	n	*1.0 μM* (% ± s.e.)	n
$\alpha 2\beta 2$	83.1 ± 3.2	6	64.4 ± 7.5	5	54.2 ± 4.0	8
$\alpha 3\beta 2$	47.2 ± 5.1	5	38.1 ± 0.9	4	30.0 ± 3.7	10
$\alpha 4$-$1\beta 2$	126 ± 12.8	8	92.8 ± 4.4	4	106.1 ± 1.5	3
$\alpha 4$-$2\beta 2$	132 ± 10.3	4	99.8 ± 10.7	3	—	—

The current responses to nicotine were expressed as a percentage of the current responses to an equal concentration of acetylcholine (ACh) applied to the same oocyte. Each row shows the mean of these responses for oocytes injected with a particular mRNA combination. The % means (± s.e.) are given at three agonist concentrations. These results have been included among the more extensive data shown graphically in Figure 2 (n = the number of oocytes from which paired responses to nicotine and ACh were obtained).

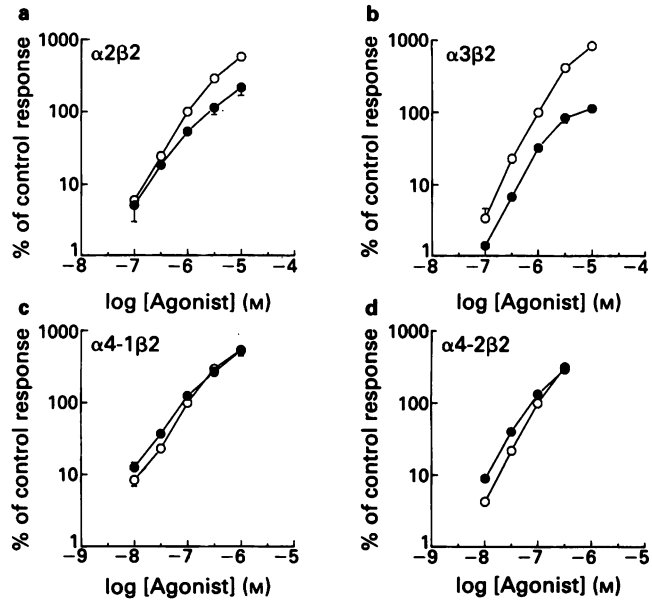

Figure 3 Log-log plots of the normalised, agonist concentration-current response relationships of oocytes injected with (a) $\alpha 2\beta 2$, (b) $\alpha 3\beta 2$, (c) $\alpha 4$-$1\beta 2$ and (d) $\alpha 4$-$2\beta 2$ RNAs. Each data point for acetylcholine (○) or nicotine (●) represents the results averaged from a minimum of 4 oocytes, except for (d) where the minimum is 3. The oocytes were voltage-clamped at -70 mV. Vertical bars, where visible outside the plot symbols, show standard errors. The current values (± s.e.) corresponding to the 100% (control) response were (a) 101.9 ± 16.2 nA, $n = 11$ oocytes; (b) 126.6 ± 28 nA, $n = 16$ oocytes; (c) 84.5 ± 18.5 nA, $n = 19$ oocytes and (d) 170.8 ± 66.6 nA, $n = 6$ oocytes. A summary of these neuronal ACh receptor dose-response characteristics is shown in Table 1.

Interaction of psychoactive drugs with neuronal nicotinic acetylcholine receptors

We took advantage of the accessibility of neuronal ACh receptors expressed in oocytes to screen the actions of several psychoactive drugs on the four neuronal subtypes described above. Representative responses shown in Figures 4 and 5, and their means are summarized in Table 3. Apart from known nicotinic antagonists, we examined a selection of clinically important drugs which, although targeted at other receptor systems, also exhibit 'anti-cholinoceptor' side effects.

Figure 4a shows the blocking of the $\alpha 2\beta 2$ receptor by mecamylamine. This drug is a potent antagonist of nicotinic ACh receptors in ganglia (Ascher *et al.*, 1979), but has limited clinical application since it crosses the blood-brain barrier and causes psychotic-like side effects. The chick $\alpha 4$ nonα-1 receptor has also been shown to be blocked by mecamylamine in a voltage independent manner (Bertrand *et al.*, 1990).

Figure 4b shows the blocking action of phencyclidine (PCP) on the $\alpha 3\beta 2$ subtype. Its non-competitive blocking action on the muscle type nicotinic ACh receptors in C2 myotubes (Changeux *et al.*, 1986) and BC3H1 cells (Albuquerque *et al.*, 1980; Papke & Oswald, 1989) has also been documented. Our results show that it also blocks central neuronal nicotinic ACh receptors (Table 3).

The major therapeutic disadvantage of amitriptyline (Ami) lies in its strong anticholinoceptor side effects. We found it was able to block neuronal nicotinic ACh receptors (Figure 4c, Table 3). At 10 μM Ami, the peak of the inhibited response is 48% of the corresponding control current value. However, if the degree of block is examined at a later time point in the

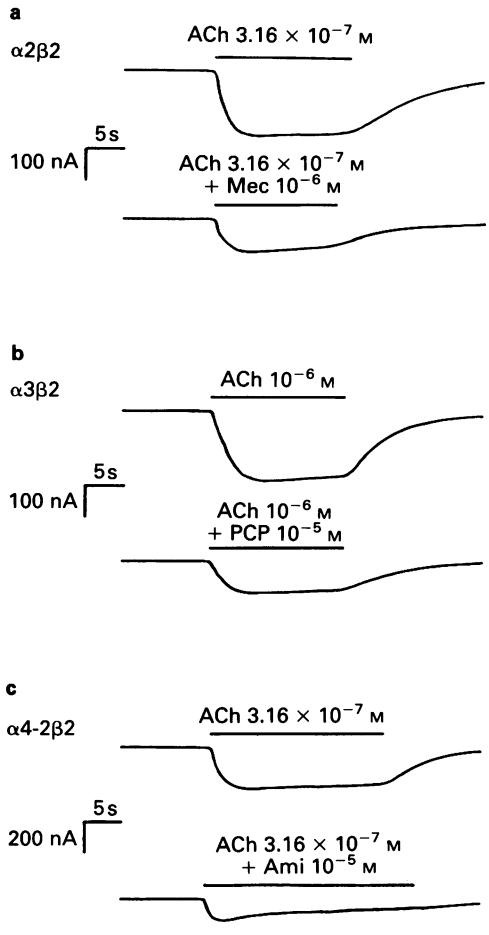

Figure 4 (a) The upper trace shows the current response to acetylcholine (ACh) of oocytes injected with α2 + β2 RNAs. The response is inhibited when both mecamylamine (Mec) and acetylcholine are applied (lower trace). (b) The control response to ACh (upper trace) of oocytes injected with α3 + β2 DNAs is reduced in the presence of phencyclidine (PCP). (c) Amitriptyline (Ami) inhibits the α4-2β2 response to acetylcholine. Note the increase in the degree of block with duration of application of the drug. All antagonists were preincubated with the oocytes for 1 min before application of the acetylcholine/antagonist mixture. The cells were voltage clamped at −70 mV. A summary of these results is shown in Table 3.

trace, then the current has diminished further (only about 32% of the control response is remaining 10 s after the start of drug application). The mechanism for this increased reduction in response is not clear, although it has been shown that the related antidepressant desipramine is an open-channel blocker of NMDA receptors and has a similar IC_{50} (Sernagor *et al.*, 1989). In those experiments shown in Table 3 where identical concentrations of agonist and antagonist were used, there was no significant difference between the subtypes at the 15% level. Therefore these antagonists do not readily discriminate between the subtypes. Also, the degree of inhibition of the peak response is remarkably similar for all the antagonists applied at a concentration of 10 μM.

Comparison of the actions of trifluoperazine and promethazine on acetylcholine receptors

The two antipsychotic drugs, trifluoperazine (TFP) and its related phenothiazine, chlorpromazine, have long been known to have an anti-nicotinic effect on *Torpedo* (Heidemann *et al.*, 1986) and peripheral ACh receptors (Changeux *et al.*, 1986). Giraudat *et al.* (1986, 1987) and Revah *et al.* (1990) have additionally found that chlorpromazine binds to amino acids in the M2 domain of the β, γ and δ subunits, a region that is thought to line the wall of the ion channel (Imoto *et al.*, 1988). The chlorpromazine binding is PCP-sensitive. Similar amino

Table 3 Inhibition of nicotinic ACh receptor response to acetylcholine by centrally active antagonists

cRNAs injected	*Antagonist*	[*ACh*] (μM)	[*Antag.*] (μM)	*% control response* (± s.e.)	n
α4-2β2	Amitriptyline	0.316	10.0	48.5 ± 7.1	3
	Promethazine	0.032	10.0	45.4 ± 0.7	3
	Mecamylamine	0.316	1.0	45.2 ± 2.3	3
	Trifluoperazine	0.316	10.0	40.0 ± 4.1	3
	Phencyclidine	0.316	10.0	34.8 ± 2.0	3
α3β2	Amitriptyline	1.0	10.0	51.0 ± 2.2	3
	Promethazine	1.0	10.0	59.9 ± 1.5	3
	Mecamylamine	1.0	1.0	62.5 ± 5.3	3
	Trifluoperazine	1.0	10.0	52.2 ± 1.6	3
	Phencyclidine	1.0	10.0	48.3 ± 2.2	3
α2β2	Amitriptyline	0.316	10.0	40.6 ± 0.6	3
	Promethazine	0.316	10.0	53.7 ± 2.0	4
	Mecamylamine	0.316	1.0	50.5 ± 0.7	3
	Trifluoperazine	0.316	10.0	47.8 ± 3.8	3
	Phencyclidine	0.316	10.0	44.6 ± 4.1	4

Initially a reproducible peak current response was obtained to the application of a control concentration of acetylcholine (ACh) (left column). Then, following a preincubation of 1 min with the antagonist, the oocytes were perfused with a mixture containing the control ACh concentration and the indicated antagonist concentration. The peak response in the presence of antagonist was divided by the control peak response and multiplied by 100 to give the values in the 5th column. The oocytes were voltage-clamped at −70 mV (n = number of oocytes in which experiments were performed).

acid residues are present in homologous positions in all the cloned neuronal ACh receptor subunits used here. We therefore tested whether receptors formed from cloned subunits of central nicotinic ACh receptors would be blocked by this TFP to see if the anti-nicotinic actions of this drug might be correlated with its clinical effects.

As a partial control for this study, the drug promethazine (Prometh) was chosen. Like TFP, the chemical structure of Prometh is also based on the phenothiazine nucleus. However Prometh lacks antipsychotic activity. If the degree of nicotinic block produced by Prometh is the same as the degree of block produced by TFP, then it would not be probable that nicotinic ACh receptors contributed to the antipsychotic effect of TFP or its motor side effects. The correlation between anti-nicotinic activity and antipsychotic activity would be negated by such a result.

The degree of blocking of the response of the α4-1β2 combination to 0.1 μM ACh by TFP and Prometh was measured over a concentration range 0.316 μM to 10 μM for these antagonists. The % of the response remaining in the presence of antagonist was assessed at two time points, firstly at the initial peak of the response, and secondly 15 s after the ACh/antagonist mixture was applied. If Figure 5a is compared with Figure 5b, then it is clear that Prometh does not show the same kind of time-dependent block as TFP. This interpretation is born out by the plots in 5C and 5D from which it is clear that the degree of block by TFP is much greater than that seen with Prometh at the 15 s time point. Similar results were also obtained with the α2β2 and α3β2 subtypes. For Prometh, the % blocks of the responses to ACh after 15 s drug application were 55.0 ± 3.0% and 60.1 ± 1.5% respectively. This is not significantly greater than the inhibition observed at the initial peaks of the responses (see Table 3). However TFP does show an increased block for the α2β2 and α3β2 combinations at the 15 s time point (34.5 ± 3.7%, 33.9 ± 3.4% respectively). In bovine chromaffin cells there is also a time-dependent increase in the degree of block by TFP (Clapham & Neher, 1984). However, unlike the latter study, the current responses obtained here in oocytes showed complete recovery, although the time course of recovery of the peak response to its initial value before antagonist application was very slow

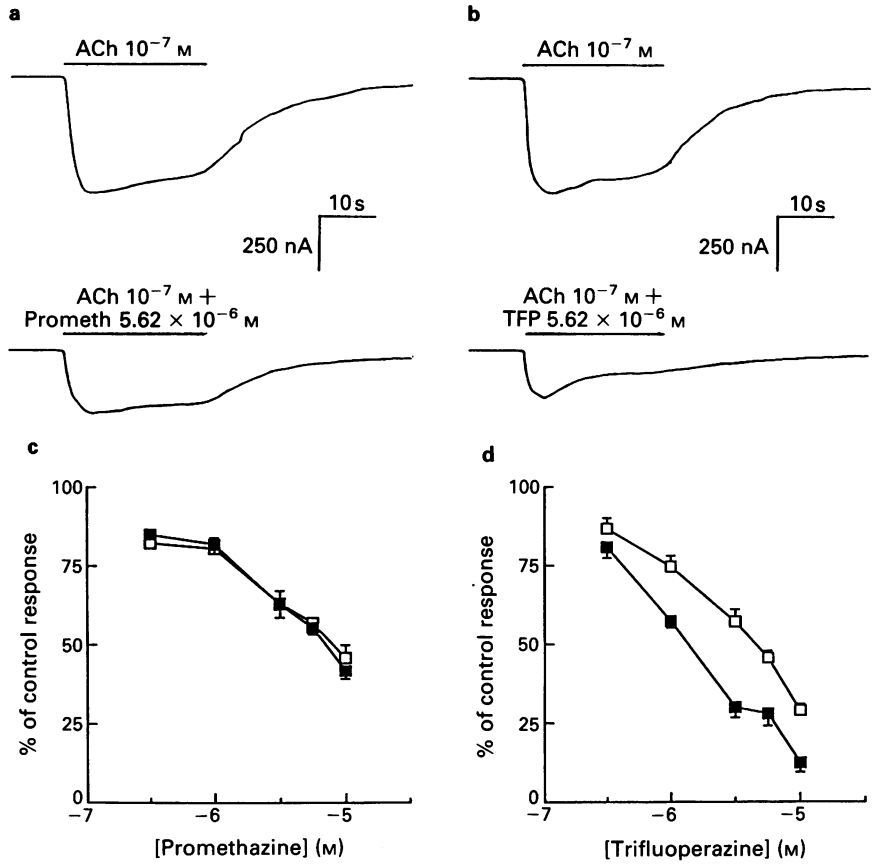

Figure 5 (a and b) The control responses of oocytes injected with the α4-1β2 RNAs to acetylcholine (ACh) are shown in the upper traces. Below them in the lower traces are the inhibited responses obtained in the presence of the antagonists promethazine (Prometh) (a) and trifluoperazine (TFP) (b). The oocytes were preincubated with the antagonists for 1 min before the application of the ACh/antagonist mixture. Note that with TFP in (b), there is a time-dependent increase in the degree of block when it is compared with the control response. (c and d) The concentration-dependent increase in the degree of block is plotted for promethazine in (c) and trifluoperazine in (d). The response remaining in the presence of antagonist is expressed as a % of the control response to 0.1 μM ACh. The degrees of inhibition at the peak of the inhibited response (□) and at a time point 15 s after the start of the response (■) were plotted. Each point represents the mean of data obtained from at least 4 oocytes. Vertical bars represent standard errors. The oocytes were voltage-clamped at −70 mV.

(longer than 20 min). Since the degree of block produced by TFP is much greater than that of Prometh, these results are consistent with the idea that the anti-nicotinic actions of TFP may contribute to its central effects.

Discussion

Functional expression of the α4-2β2 neuronal nicotinic acetylcholine receptors

Here we report the functional expression of the rat α4-2 subunit in conjunction with the β2 subunit. The experiments we have performed do not reveal any significant functional distinction between the α4-1β2 and α4-2β2 subtypes. This might be expected, given the position and size of the altered amino acid sequence involved. In rat brain, α4-2 mRNAs are slightly more abundant than α4-1 RNAs (Goldman *et al.*, 1987), although *in situ* hybridization studies show that they share an almost identical pattern of distribution (Wada *et al.*, 1988a). However, these results do not rule out the possibility that the different C-termini are related to some other property of the receptors such as recognition motif for incoming nerve fibres. It is interesting to note that the rat α2 subunit also ends in the sequence GMI, although there is no evidence of an additional splicing variant of α2 in rat. The GMI sequence is also found at the C-terminal of the chick α2 and α4 subunits, although neither subunit has splicing variants in this species (Nef *et al.*, 1988).

The *in situ* hybridisation study described above additionally showed that the distribution patterns of the α4 transcripts overlap with that of the β2 transcripts and also coincide with many of the brain regions previously associated with high-affinity nicotine binding (Clarke *et al.*, 1985). N-terminal sequencing of immuno-affinity purified, neuronal ACh receptors reveals that the predominant subunit ACh receptor species in brain are α4 and β2 subunits (Whiting *et al.*, 1987a; Schoepfer *et al.*, 1988). It was further found that the α4 subunit binds nicotine with nanomolar affinity (Whiting *et al.*, 1987b). However, since the N-termini of the rat α4-1 and α4-2 subunits are identical, it is not possible to determine the relative proportions of the α4-1 and α4-2 subunit proteins by this method. An antibody directed against β2 subunits has been shown to bind extensively throughout the brain and in many presynaptic locations (Swanson *et al.*, 1987). This additional evidence, coupled to the present demonstration that the α4-2β2 combination is functional, makes it probable both α4-1β2 and α4-2β2 are subunit combinations present in nicotinic ACh receptors in mammalian brain, possibly in presynaptic locations. It is also possible that both the α4-1 and α4-2 subunits might be found in the same brain receptor.

Differential sensitivity of nicotinic acetylcholine receptors to nicotine

There is a clear difference between muscle and neuronal receptors in their relative sensitivity to nicotine as compared to ACh. Muscle receptors are at least 50 times less sensitive than neuronal receptors at low agonist concentrations. There are also differences among the neuronal receptors. The α4-1β2 and α4-2β2 combinations are the most sensitive to nicotine. A

similar degree of sensitivity to nicotine has been seen for the chick combination of $\alpha4\beta2$ (Bertrand *et al.*, 1990), for which a nicotine EC_{50} of 0.77 μM was reported. The rat $\alpha2\beta2$ combination was less sensitive than these, while the $\alpha3\beta2$ combination was the least sensitive of the neuronal receptors. Overall, the relative nicotine sensitivities of ACh receptor subtypes studied in this present work (compared with their response to 0.1 μM ACh and presented in the order of decreasing sensitivity) were: $\alpha4$-$1\beta2$, (126) = $\alpha4$-$2\beta2$, (126) > $\alpha2$-$2\beta2$, (95.1) > $\alpha3\beta2$, (47.8) > $\alpha1\beta1\gamma\delta$, (1).

Recently, Leutje & Patrick (1991) have also examined the relative nicotine sensitivity of the cloned receptors and have observed a similar difference in potency between muscle and neuronal nicotinic ACh receptors. There is broad agreement between their results for neuronal receptors and those described here, except in the case of the $\alpha2\beta2$ combination. They report a nicotine: ACh current response ratio greater than 5:1 for agonist concentrations of 3 μM, whereas our results give an estimated ratio of 0.4:1 at that concentration. The time interval required between agonist applications for recovery of the peak current response suggests that there is considerable desensitization of these receptors at μM concentrations (see Methods). For muscle receptors, the time constant for this desensitization is less than 100 ms (Dilger & Brett, 1990; Franke *et al.*, 1991). The rate of drug application was slightly slower in the Leutje & Patrick study than that used here, and intracellular Ca^{2+} concentrations were not buffered. Therefore one possible explanation for the discrepancy between the two sets of observations is that the different conditions could give rise to different degrees of desensitization at the observed peak responses if the rates of desensitization associated with the two drugs are different. None the less, both sets of studies do illustrate the great sensitivity of the neuronal receptors to nicotine.

Despite this sensitivity, the results described here and for the chick $\alpha4\beta2$ combination are not consistent wtih K_d estimates from binding studies of 1–10 nM for the high affinity (−)-nicotine binding site in rat brain (Wonnacott *et al.*, 1990, review). However, the equilibrium conditions under which binding studies are carried out often involve prolonged exposure of the receptors to high concentrations of agonist. This favours desensitization, in which state the receptor may be expected to have a higher affinity for nicotine than during the less desensitizing conditions of electrophysiological assays.

The above results suggest that the subunits we have expressed may mediate many of the effects of nicotine. However, it should be noted that the recently cloned chick $\alpha7$ subunit is also very sensitive to nicotine (Couturier *et al.*, 1990b). In addition, studies in *Xenopus* oocytes suggest that neuronal ACh receptors incorporating the rat $\beta4$ subunit will have different properties, including enhanced nicotine sensitivity, when compared with receptors in which $\beta2$ is the only non-α subunit (Duvoisin *et al.*, 1989; Leutje & Patrick, 1991; Papke & Heinemann, 1991). This is also true for the equivalent subunits in chick (Couturier *et al.*, 1990a). Therefore the total response of the brain to nicotine will involve more subunits than those examined here. These considerations, desensitization, and the possible involvement of uncloned subunits, make nicotine sensitivity difficult to use, at present, as a test to identify and discriminate between subtypes in the nervous system.

Sensitivity of neuronal acetylcholine receptors to psychoactive drugs

It is interesting to note that despite their structural diversity, all the psychoactive antagonists tested were effective blockers of neuronal nicotinic ACh receptors. The most potent of these antagonists was mecamylamine. The estimated equilibrium constant of mecamylamine for ACh receptors in ganglia is 50 nM (Ascher *et al.*, 1979) and an IC_{50} of 200 nM has been observed in PC12 cells (Lukas, 1989). This is somewhat lower than the value of approximately 1 μM suggested by the results obtained here (Table 3). The difference may arise because we have not fully reproduced the pharmacological properties of native ganglionic receptors with the combinations we have introduced into oocytes. However, for combinations which are representative of native receptors, the pharmacological properties of these receptors are expected to be essentially identical to those of equivalent receptors found in man. This is because human muscle (Luther *et al.*, 1989) and neuronal ($\alpha3$: Fornasari *et al.*, 1990; $\beta2$: Anand & Lindstrom, 1990) nicotinic ACh receptor clones encode proteins with a very high sequence identity with the equivalent rat subunits. Studies such as these may therefore be useful in addressing drug design problems.

The potent inhibitory action of the tricyclic antidepressant, amitryptiline, on the nicotinic receptors expressed here illustrates this point. Amitriptyline has a tendency to induce weight gain (Vendsborg *et al.*, 1976) whereas nicotine addition tends to inhibit it (Wack & Rodin, 1982). It therefore seems possible that an anti-nicotinic action of amitriptyline could be responsible for its weight gain side-effect. However, as noted above, the related antidepressant desipramine blocks NMDA receptors (Sernagor *et al.*, 1989).

NMDA receptors are also blocked by mecamylamine (O'Dell & Christensen, 1988) and PCP (MacDonald *et al.*, 1987). The observation that (+)-tubocurarine and MK-801 block both NMDA receptors (MacDonald *et al.*, 1987; Halliwell *et al.*, 1989) and nicotinic ACh receptors (Ascher *et al.*, 1979; Kavanaugh *et al.*, 1989; Amador & Dani, 1991) adds to the pharmacological similarity of these ion channels. Interestingly, (+)-tubocurarine, cocaine and ondansetron have also been shown to block both 5-HT_3 receptors (Higashi & Nishi, 1982; Richardson *et al.*, 1985; Yakel & Jackson, 1988; Peters *et al.*, 1990) and nicotinic ACh receptors (Bertrand *et al.*, 1990; Vanner & Suprenant, 1990). However, ondansetron is 100–1000 times more potent a blocker of 5-HT_3 receptors than of nicotinic ACh receptors and 5-HT_3 receptors are relatively insensitive to the ganglionic nicotinic antagonists, hexamethonium (Vanner & Suprenant, 1990) and trimetaphan (Peters *et al.*, 1990), and voltage recordings suggest that the block of these receptors by (+)-tubocurarine is voltage-independent (Vanner & Suprenant, 1990). Despite these differences between the receptor classes, the pharmacological, similarity is still strong enough to add to the difficulty of interpreting electrophysiological and binding studies in neuronal tissue. However, perhaps the most remarkable aspect of the observations described here is the similarity in the degree of block produced by these varied compounds.

Trifluoperazine is a potent blocker of central neuronal nicotinic acetylcholine receptors

Pharmacological studies of TFP and other drugs used to relieve the symptoms of schizophrenia have correlated their anti-psycholtic potency with their anti-dopaminergic potency (Creese *et al.*, 1976; Seeman *et al.*, 1976). Unlike TFP, Prometh does not have antipsychotic activity and does not inhibit vasopressin release (Dyball *et al.*, 1968) (a hormone whose circulating levels are increased by nicotine administration, Seyler *et al.*, 1986). These results suggest that Prometh may be less anti-nicotinic than TFP.

We have seen above that 3.16 μM TFP can diminish the ACh response of neuronal nicotinic ACh receptors to about 25% of its control value. Prometh will only inhibit ACh receptors to about 60% of their control responses, and does not show a time-dependent increase in block. Therefore, our results suggest that in the brain there could be considerable, selective inhibition of central nicotinic ACh receptors at therapeutic concentrations of TFP (0.5–5.0 μM). The existence of excitatory presynaptic nicotinic receptors, which can elicit dopamine release, has been shown in several studies (Connelly & Littleton, 1983; Takano *et al.*, 1983; Schulz & Zigmond, 1989; Rapier *et al.*, 1990; Brazell *et al.*, 1990). It therefore

seems possible that TFP could block presynaptic nicotinic ACh receptors and diminish their contribution to dopamine release. This would help limit the number of postsynaptic dopamine receptors that are activated, augmenting the drug's postsynaptic blocking action associated with its antipsychotic activity. Alternatively, nicotinic receptor blockade by antipsychotic drugs may be more important in the development of side effects such as weight gain, inappropriate vasopressin release syndrome and tardive dyskinesia. It has been noted that smokers have a reduced risk of suffering from Parkinson's disease (Baron, 1986) and a protective effect for nicotine has been suggested by Moss *et al.* (1989) who have found it to be an effective adjunct to neuroleptic treatment for the reduction of motor tics in Tourettes Syndrome.

The present results show the potential usefulness of pharmacological studies on defined subunit combinations for identifying novel targets for therapeutic intervention and improving the understanding of the mechanisms by which illnesses produce their symptoms. In addition, the present study demonstrates that it is feasible that neuronal nicotinic ACh receptors make a significant contribution to the therapeutic and adverse actions of psychoactive drugs in man.

The authors would especially like to thank Karin Doebeli and Anne O'Shea-Greenfield for excellent technical assistance. They are also grateful to Prof. David Colquhoun, Prof. Don Jenkinson, Dr Alasdair Gibb, Dr Mark Farrant and Jeff Dalley for helpful discussions and critical reading of the manuscript. This work was supported by grants to S.H. from NINCDS, (NS11549), the Muscular Dystrophy Association (U.S.A.), and the Cigarette and Tobacco Surtax Fund of the State of California, and a Postdoctoral Fellowship to J.C. from the Muscular Dystrophy Association (U.S.A.).

References

ALBUQUERQUE, E.X., TSAI, M.-C., ARONSTAM, R.S., ELDERFRAWI, A.T. & ELDERFRAWI, M.E. (1980). Sites of action of phenycyclidine II. Interaction with the ionic channel of the nicotinic acetylcholine receptor. *Mol. Pharmacol.*, **18**, 167–178.

AMADOR, M. & DANI, J.A. (1991). MK-801 Inhibition of nicotinic acetylcholine receptor channels. *Synapse*, **7**, 207–215.

ANAND, R. & LINDSTROM, J. (1990). Nucleotide sequence of the human nicotinic acetylcholine receptor beta 2 subunit gene. *Nucleic Acids Res.*, **18**, 4272.

ASCHER, P., LARGE, W.A. & RANG, H.P. (1979). Studies on the mechanism of action of acetylcholine antagonists on rat parasympathetic ganglion cells. *J. Physiol.*, **295**, 139–170.

BALLIVET, M., NEF, P., COUTUREIER, S., RUNGGER, D., BADER, C.R., BERTRAND, D. & COOPER, E. (1988). Electrophysiology of a chick neuronal nicotinic acetylcholine receptor expressed in *Xenopus* oocytes after cDNA injection. *Neuron*, **1**, 847–852.

BARON, J.A. (1986). Cigarette smoking and Parkinson's disease. *Neurology*, **36**, 1490–1496.

BENOWITZ, N.L. (1988). Pharmacological aspects of cigarette smoking and nicotine addiction. *New Engl. J. Med.*, **319**, 1318–1330.

BERTRAND, D., BALLIVET, M. & RUNGGER, D. (1990). Activation and blocking of a neuronal nicotinic acetylcholine receptor reconstituted in *Xenopus* oocytes. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 1993–1997.

BOULTER, J., EVANS, K., GOLDMAN, D., MARTIN, G., TRECO, D., HEINEMANN, S. & PATRICK, J. (1986a). Isolation of a cDNA clone coding for a possible neural nicotinic acetylcholine receptor alpha-subunit. *Nature*, **319**, 368–374.

BOULTER, J.B., EVANS, K., MARTIN, G., MASON, P., STENGELIN, S., GOLDMAN, D., HEINEMANN, S. & PATRICK, J. (1986b). Isolation and sequence of cDNA clones coding for the precursor to the gamma subunit of mouse muscle nicotinic acetylcholine receptor. *J. Neurosci. Res.*, **16**, 37–49.

BOULTER, J., CONNOLLY, J.G., DENERIS, E., GOLDMAN, D., HEINEMANN, S. & PATRICK, J. (1987). Functional expression of two neuronal nicotinic acetylcholine receptors from cDNA clones identifies a gene family. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 7763–7767.

BOULTER, J., LUYTEN, W., EVANS, K., MASON, P., BALLIVET, M., GOLDMAN, D., STENGELIN, S., MARTIN, G., HEINEMANN, S. & PATRICK, J. (1985). Isolation of a clone coding for the alpha-subunit of a mouse nicotinic acetylcholine receptor. *J. Neurosci.*, **5**, 2545–2552.

BOULTER, J., O'SHEA-GREENFIELD, A., DUVOISIN, R.M., CONNOLLY, J.G., WADA, E., JENSEN, A., MACKINNON, D., BALLIVET, M., DENERIS, E.S., HEINEMANN, S. & PATRICK, J. (1990). $\alpha 3$, $\alpha 5$ and $\beta 4$, three members of a rat neuronal nicotinic acetylcholine receptor related gene family form a gene cluster. *J. Biol. Chem.*, **265**, 4472–4482.

BRAZELL, M.P., MITCHELL, S.N., JOSEPH, M.H. & GRAY, J.A. (1990). Acute administration of nicotine increases the *in vivo* extracellular levels of dopamine, 3,4-dihydroxyphenylacetic acid and ascorbic acid preferentially in the nucleus accumbens of the rat: comparison with caudate putamen. *Neuropharmacology*, **26**, 1177–1185.

BULLER, A.L. & WHITE, M.M. (1990). Functional acetylcholine receptors expressed in *Xenopus* oocytes after injection of *Torpedo*, β, γ and δ subunit mRNAs are a consequence of endogenous gene expression. *Mol. Pharmacol.*, **37**, 423–428.

CHANGEUX, J.P., PINSET, C. & RIBERA, A.B. (1986). Effects of chlorpromazine and phenycyclidine on mouse C2 acetylcholine receptor kinetics. *J. Physiol.*, **378**, 497–513.

CLAPHAM, D.E. & NEHER, E. (1984). Trifluoperazine reduces inward ionic currents and secretion by separate mechanisms in bovine chromaffin cells. *J. Physiol.*, **353**, 541–564.

CLARKE, P.B.S., SCHWARTZ, R.D., PAUL, S.M., PERT, C.B. & PERT, A. (1985). Nicotinic binding in the rat brain: Autoradiographic comparison of [^{3}H]-acetylcholine, [^{3}H]-nicotine and [^{125}I]-alpha-bungarotoxin. *J. Neurosci.*, **5**, 1307–1315.

CONNELLY, N.S. & LITTLETON, J.M. (1983). Lack of stereoselectivity in ability of nicotine to release dopamine from rat synaptosomal preparations. *J. Neurochem.*, **41**, 1297–1302.

CONNOLLY, J.G. (1989). Structure-function relationships in nicotinic acetylcholine receptors. *Comp. Biochem. Physiol.*, **93A**, 221–231.

CONTI-TRONCONI, B.M., DUNN, S.M.J., BARNARD, E.A., DOLLY, J.O., LAI, F.A., RAY, N. & RAFTERY, M.A. (1985). Brain and muscle nicotinic acetylcholine receptors are different but homologous proteins. *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 5208–5212.

COOPER, E., COUTURIER, S. & BALLIVET, M. (1991). Pentameric structure and subunit stoichiometry of a neuronal nicotinic acetylcholine receptor. *Nature*, **350**, 235–238.

COUTURIER, S., BERTRAND, D., MATTER, J.-M., HERNANDEZ, M.-C., BERTRAND, S., MILLAR, N., VALERA, S., BARKAS, T. & BALLIVET, M. (1990a). A neuronal nicotinic acetylcholine receptor subunit ($\alpha 7$) is developmentally regulated and forms a homo-oligomeric channel blocked by α-bungarotoxin. *Neuron*, **5**, 847–856.

COUTURIER, S., ERKMAN, L., VALERA, S., RUNGGER, D., BERTRAND, S., BOULTER, J., BALLIVET, M. & BERTRAND, D. (1990b). $\alpha 5$, $\alpha 3$ and non-$\alpha 3$, three clustered avian genes encoding neuronal nicotinic acetylcholine receptor-related subunits. *J. Biol. Chem.*, **265**, 17560–17567.

CREESE, I., BURT, D.R. & SNYDER, S.H. (1976). Dopamine receptor binding predicts clinical and pharmacological potencies of antipsychotic drugs. *Science*, **192**, 481–83.

CURTIS, D.R. & ECCLES, R.M. (1958). The excitation of Renshaw cells by pharmacological agents applied electrophoretically. *J. Physiol.*, **141**, 435–445.

DENERIS, E., CONNOLLY, J., BOULTER, J., WADA, K., WADA, E., SWANSON, L., PATRICK, J. & HEINEMANN, S. (1988). Identification of a cDNA coding for a subunit common to distinct acetylcholine receptors. *Neuron*, **1**, 45–54.

DENERIS, E.S., CONNOLLY, J.G., ROGERS, S.W. & DUVOISIN, R.M. (1991). Pharmacological and functional diversity of neuronal nicotinic AChRs. *Trends Pharmacol. Sci.*, **12**, 34–40.

DILGER, J.P. & BRETT, R.S. (1990). Direct measurement of the concentration- and time-dependent open probability of the nicotinic acetylcholine receptor channel. *Biophys. J.*, 723–731.

DUVOISIN, R.M., DENERIS, E.S., PATRICK, J. & HEINEMANN, S. (1989). The functional diversity of neuronal nicotinic acetylcholine receptors is increased by a novel subunit: $\beta 4$. *Neuron*, **3**, 589–596.

DYBALL, R.E. (1968). The effects of drugs on the release of vasopressin. *Br. J. Pharmacol.*, **33**, 29–41.

FORNASARI, B., CHINI, P., TARRONI, P. & CLEMENTI, F. (1990). Molecular cloning of human neuronal nicotinic receptor $\alpha 3$-subunit. *Neurosci. Lett.*, **111**, 351–356.

FRANKE, C., HATT, H. & DUDEL, H. (1991). Steep concentration dependence and fast desensitization of nicotinic channel currents

elicited by acetylcholine pulses, studies in adult vertebrate muscle. *Pflügers Arch.-Eur. J. Physiol.*, **417**, 509–527.

GIRAUDAT, J., DENNIS, M., HEIDMANN, T., CHANG, J.Y. & CHANGEAX, J.P. (1986). Structure of the high affinity binding site for noncompetitive blockers of the acetylcholine receptor: Serine-262 of the delta subunit is labelled by [^{3}H]chlorpromazine. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 2719–2723.

GIRAUDAT, J., DENNIS, M., HEIDMANN, T., HAUMONT, P.T., LEDERER, P. & CHANGEUX J.P. (1987). Structure of the high affinity site for noncompetitive blockers of the acetylcholine receptor: [^{3}H]-chlorpromazine labels homologous residues in the beta and delta chains. *Biochemistry*, **26**, 2410–2418.

GOLDMAN, D., DENERIS, E., KOCHAR, A., PATRICK, J. & HEINEMANN, S. (1987). Members of a nicotinic acetylcholine receptor gene family are expressed in different regions of the mammalian central nervous system. *Cell*, **48**, 965–973.

HALLIWELL, R.F., PETERS, J.A. & LAMBERT, J.J. (1989). The mechanism of action and pharmacological specificity of the anticonvulsant NMDA antagonist MK801: a voltage clamp study on neuronal cells in culture. *Br. J. Pharmacol.*, **96**, 480–494.

HARTMAN, D.S. & CLAUDIO, T. (1990). Coexpression of two distinct muscle acetylcholine receptor α-subunits during development. *Nature*, **343**, 372–375.

HEIDEMANN, T., OSWALD, R.E. & CHANGEUX, J.P. (1986). Multiple sites of action for noncompetitive blockers of acetylcholine, receptors rich membrane fragments from *Torpedo marmorata*. *Biochemistry*, **22**, 3112–3127.

HEINEMANN, S., BOULTER, J., CONNOLLY, J., GOLDMAN, D., EVANS, K., TRECO, D., BALLIVET, M. & PATRICK, J. (1986). In *Nicotinic Acetylcholine Receptor: Structure and Function* ed. Maelicke, A. pp. 359–389. Nato ASI series: Berlin: Springer-Verlag.

HIGASHI, H. & NISHI, S. (1982). 5-Hydroxytryptamine receptors of visceral primary afferent neurones on rabbit nodose ganglia. *J. Physiol.*, **323**, 543–567.

HILL, A.V. (1909). The mode of action of nicotine and curari, determined by the form of the contraction curve and the method of temperature coefficients. *J. Physiol.*, **39**, 361–373.

IMOTO, K., BUSCH, C., SAKMANN, B., MISHINA, M., KONNO, T., NAKAI, J., BUJO, H., MORI, Y., FUKUDA, K. & NUMA, S. (1988). Rings of negatively charged as determine the AChR channel conductance. *Nature*, **335**, 643–648.

KAO, P.N., DWORK, A.J., KALDANEY, R.J., SILVER, M.L., WIDEMAN, J., STEIN, S. & KARLIN, A. (1984). Identification of two alpha-subunit half-cysteines specifically labelled by an affinity reagent for the acetylcholine binding site. *J. Biol. Chem.*, **259**, 1162–1165.

KAVANAUGH, M.P., TESTER, B.A. & WEBER, E. (1989). Interaction of MK-801 with the nicotinic acetylcholine receptor-associated ion channel from electroplax. *Eur. J. Pharmacol.*, **164**, 397–398.

KUROSAKI, T., FUKUDA, K., KONNO, T., MORI, Y., TANAKA, K.-I., MISHINA, M. & NUMA, S. (1987). Functional properties of nicotinic acetylcholine receptor subunits expressed in various combinations. *F.E.B.S. Lett.*, **214**, 253–258.

LEUTJE, C.W. & PATRICK, J. (1991). Both α- and β- subunits contribute to the agonist sensitivity of neuronal nicotinic acetylcholine receptors. *Neuroscience*, **11**, 837–845.

LUKAS, R.J. (1989). Pharmacological distinctions between the functional nicotinic acetylcholine receptors on the PC12 rat pheochromocytoma and the rat TE671 human medulloblastoma. *J. Pharmacol. Exp. Ther.*, **251**, 175–182.

LUTHER, M.A., SCHOEPFER, R., WHITING, P., CASEY, B., BLATT, Y., MONTAL, M.S., MONTAL, M. & LINDSTROM, J. (1989). A muscle acetylcholine receptor is expressed in the human cerebellar medulloblastoma cell line TE671. *J. Neurosci.*, **9**, 1082–1096.

MACDONALD, J.F., MILJKOVIC, Z. & PENNEFATHER, P. (1987). Use-dependent block of excitatory amino acid currents in cultured neurons by ketamine. *J. Neurophysiol.*, **58**, 251–266.

MELTON, D.A., KRIEG, P.A., REBAGLIATI, M.R., MANIATIS, T., ZINN, K. & GREEN, M.R. (1984). Efficient *in vivo* synthesis of biologically active RNA and RNA hybridisation probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.*, **12**, 7035–7056.

MOSS, D.E., MERSCHEID, P.A., MONTGOMERY, S.P., NORMAN, A.B. & SANBERG, P.R. (1989). Nicotine and cannabinoids as adjuncts to neuroleptics in the treatment of Tourette syndrome and other motor disorders. *Life Sci.*, **44**, 1521–1525.

NEF, P., ONESYER, C., ALLIOD, C., COUTURIER, S. & BALLIVET, M. (1988). Genes expressed in the brain define three distinct neuronal nicotinic acetylcholine receptors. *EMBO*, **7**, 7595–7601.

O'DELL, T.J. & CHRISTENSEN, B.N. (1988). Mecamylamine is a selective non-competitive antagonist of N-methyl-D-aspartate and aspartate induced currents in horizontal cells dissociated from the catfish retina. *Neurosci. Lett.*, **94**, 93–98.

PAPKE, R.L. & HEINEMANN, S.F. (1991). The role of the β4 subunit in determining the kinetic properties of rat neuronal nicotinic acetylcholine α3 receptors. *J. Physiol.*, **440**, 95–112.

PAPKE, R.L. & OSWALD, R.E. (1989). Mechanisms of noncompetitive inhibition of acetylcholine-induced single channel currents. *J. Gen. Physiol.*, **93**, 785–711.

PETERS, J.A., MALONE, H.M. & LAMBERT, J.J. (1990). Antagonism of 5-HT$_3$ receptor mediated currents in murine N1E-115 neuroblastoma cells by (+)-tubocurarine. *Neurosci. Lett.*, **110**, 107–112.

RAPIER, C., LUNT, G.G. & WONNACOTT, S. (1990). Nicotinic modulation of [^{3}H]dopamine release from striatal synpatosomes: pharmacological characterisation. *J. Neurochem.*, **54**, 937–945.

REVAH, F., GALZI, J.L., GIRAUDAT, J., HAUMONT, P.Y., LEDERER, F. & CHANGEUX, J.P. (1990). The noncompetitive blocker [^{3}H]chlorpromazine labels three amino acids of the acetylcholine receptor gamma subunit: implications for the alpha-helical organization of regions MII and for the strcture of the ion channel. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 4675–4679.

RICHARDSON, D.P., ENGEL, G., DONATSCH, D. & STADLER, P.A. (1985). Identification of serotonin M-receptors and their specific blockade by a new class of drugs. *Nature*, **316**, 126–131.

SCHOEPFER, R., WHITING, P., ESCH, F., BLACHER, R., SHIMASAKI, S. & LINDSTROM, J. (1988). cDNA clones coding for the structural subunit of a chicken brain nicotinic acetylcholine receptor. *Neuron*, **1**, 241–248.

SCHULZ, D.W. & ZIGMOND, R.E. (1989). Neuronal bungarotoxin blocks the nicotinic stimulation of endogenous dopamine release from rat striatum. *Neurosci. Lett.*, **98**, 310–316.

SEEMAN, P., LEE, T., CHAU-WONG, M. & WONG, K. (1976). Antipsychotic drug doses and neuroleptic/dopamine receptors. *Nature*, **262**, 717–719.

SERNAGOR, E., KUHN, D., VYLICKY, JR., L. & MAYER, M.L. (1989). Open channel block of NMDA receptor responses evoked by tricyclic antidepressants. *Neuron*, **2**, 1221–1227.

SEYLER, L.E., JR., POMERLEAU, O.F., FERTIG, J.B., HUNT, D. & PARKER, K. (1986). Pituitary hormone response to cigarette smoking. *Pharmacol. Biochem. Behav.*, **24**, 159–162.

SWANSON, L., SIMMONS, D., WHITING, P. & LINDSTROM, J. (1987). Immunohistochemical localization of neuronal nicotinic receptors in the rodent central nervous system. *J. Neurosci.*, **7**, 3334–3342.

TAKANO, Y., SAKURAI, Y., KOHJIMOTO, Y., HONDA, K. & KANIYA, H. (1983). Presynaptic modulation of the release of dopamine from striatal synaptosomes: differences in the effect of high K^+ stimulation, methamphetamine and nicotinic drugs. *Brain Res.*, **279**, 330–334.

VANNER, S. & SUPRENANT, A. (1990). Effects of 5-HT$_3$ receptor antagonists on 5-HT and nicotinic depolarisations in guinea-pig submucosal neurones. *Br. J. Pharmacol.*, **99**, 840–844.

VENDSBORG, P.B., BECH, P. & RAFAELSON, O.J. (1976). Lithium treatment and weight gain. *Acta. Psychiat. Scand.*, **53**, 139–147.

WACK, J.T. & RODIN, J. (1982). Smoking and its effect on body weight and the systems of caloric regulation. *Am. J. Clin. Nutr.*, **35**, 366–380.

WADA, K., BALLIVET, M., BOULTER, J., CONNOLLY, J., WADA, E., DENERIS, E., SWANSON, L.W., HEINEMANN, S. & PATRICK, J. (1988b). Primary structure and expression of beta-2, a novel subunit of neuronal nicotinic acetylcholine receptors. *Science*, **240**, 330–334.

WADA, E., WADA, K., BOULTER, J., DENERIS, E., HEINEMANN, S., PATRICK, J. & SWANSON, L.W. (1988a). The distribution of alpha2, alpha3, alpha4 and beta2 neuronal nicotinic acetylcholine receptor subunit mRNAs in the central nervous system: A hybridisation histochemical study in the rat. *J. Comp. Neurol.*, **284**, 314–335.

WARBURTON, D.M. (1987). The functions of smoking. In *Advances in Behavioural Biology*, vol. 31. *Tobacco Smoking and Nicotine, a Neurobiological Approach.* ed. Martin, W.R., Van Loon, G.R., Iwamoto, E.T. & Davis, L. pp. 51–61. New York: Plenum Press.

WESNES, K. & WARBURTON, D.M. (1983). Smoking, nicotine and human performance. *Pharmacol. Ther.*, **21**, 189–208.

WHITEHOUSE, P.J., MARTINO, A.M., MARCUS, K.A., ZWEIG, R.M., SINGER, H.S., PRICE, D.C. & KELLAR, K.J. (1988). Reductions in acetylcholine and nicotine binding in several degenerative diseases. *Archiv. Neurology*, **45**, 722–724.

WHITING, P., LIU, R., MORLEY, B.J. & LINDSTROM, J. (1987a). Structurally different neuronal nicotinic acetylcholine receptor subtypes purified and characterised using monoclonal antibodies. *J. Neurosci.*, **7**, 4005–4016.

WHITING, P., ESCH, F., SHIMASAKI, S. & LINDSTROM, J. (1987b). Neuronal nicotinic acetylcholine receptor beta subunit is coded for by the cDNA clone alpha4. *FEBS Lett.*, **219**, 459–463.

WONNACOTT, S., RUSSELL, M.A.H. & STOLERMAN, I.P. (1990). Characterisation of nicotine receptor sites in the brain. In *Nicotine Psychopharmacology: Molecular Cellular and Behavioural Aspects.* pp. 226–306. Oxford University Press.

YOSHI, Y., YU, L., MAYNE, K.M., DAVIDSON, N. & LESTER, H. (1987). Equilibrium properties of mouse-*Torpedo* acetylcholine receptor hybrids expressed in *Xenopus* oocytes. *J. Gen. Physiol.*, **90**, 553–573.

YAKEL, J.L. & JACKSON, M.B. (1988). 5-HT_3 receptors mediate rapid responses in cultured hippocampus and a channel cell line. *Neuron*, **1**, 615–621.

(Received June 14, 1991
Revised October 8, 1991
Accepted November 27, 1991)