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Both α- and β-subunits Contribute to the Agonist Sensitivity of Neuronal Nicotinic Acetylcholine Receptors

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A family of genes has been identified that encodes subunits of nicotinic acetylcholine receptors (nAChRs) and is expressed in the nervous system. Functional neuronal nAChRs can be expressed in *Xenopus* oocytes by injection of RNA encoding 1 of 2 different β-subunits (β2, β4) in pairwise combination with RNA encoding 1 of 3 different α-subunits (α2, α3, α4). We examined the sensitivity of these 6 different α–β-subunit combinations to the nicotinic agonists ACh, nicotine, cytisine, and 1,1-dimethyl-4-phenylpiperazinium (DMPP). Each subunit combination displayed a distinct pattern of sensitivity to these 4 agonists. The α2β2 combination was 5-fold more sensitive to nicotine than to acetylcholine, while the α3β2 combination was 17-fold less sensitive to nicotine than to ACh, and the α3β4 combination was equally sensitive to both nicotine and ACh. nAChRs composed of α2, α3, or α4 in combination with β2 were 14–100-fold less sensitive to cytisine than to ACh. In contrast, nAChRs composed of α2, α3, or α4 in combination with β4 were 3–17-fold more sensitive to cytisine than to ACh. The α2β2, α3β2, and α3β4 combinations were each equally sensitive to DMPP and ACh, while the α2β4, α4β2, and α4β4 combinations were 4–24-fold less sensitive to DMPP than to ACh. We also demonstrated that these differences are neither a consequence of variation in the relative amounts of RNA injected nor an artifact of oocyte expression. The oocyte system can accurately express ligand-gated ion channels because mouse muscle nAChRs expressed in oocytes display pharmacological properties similar to those reported for these receptors expressed on BC3H-1 cells. We conclude that both the α- and the β-subunits contribute to the pharmacological characteristics of neuronal nAChRs.

Nicotinic acetylcholine receptors (nAChRs) are present at the neuromuscular junction and throughout the CNS and PNS (for reviews, see Colquhoun et al., 1987; Luetje et al., 1990b). It has long been known that neuronal nAChRs differ from nAChRs found at the neuromuscular junction. Neuronal nAChRs display differential sensitivity to a variety of nicotinic antagonists and have distinct single-channel properties when compared with muscle nAChRs (for reviews, see Colquhoun et al., 1987; Steinbach and Ifune, 1989). In addition, neuronal nAChRs are insensitive to blockade by elapid α-neurotoxins, such as α-bungarotoxin, which potently inhibit neuromuscular nAChRs (Brown and Fumagalli, 1977; Patrick and Stallcup, 1977; Carbonetto et al., 1978).

Recent molecular cloning experiments have revealed a gene family encoding a number of neuronal nAChR subunits. At present, 3 functional α-subunits [α2 (Wada et al., 1988), α3 (Boulter et al., 1986), and α4 (Goldman et al., 1987)] and 2 functional β-subunits [β2 (Deneris et al., 1988) and β4 (Duvoisin et al., 1989)] have been described. Injection of RNA encoding either β2 or β4 into *Xenopus* oocytes in pairwise combination with RNA encoding either α2, α3, or α4 results in the expression of functional nAChRs (Boulter et al., 1987; Deneris et al., 1988; Wada et al., 1988; Duvoisin et al., 1989). Several studies of different neuronal nAChR subunit combinations expressed in oocytes have revealed a diversity of single-channel properties (Papke et al., 1989a,b) and differences in sensitivity to the nicotinic antagonist dihydro-β-erythroidine and to several neurotoxins (Duvoisin et al., 1989; Luetje and Patrick, 1989; Luetje et al., 1990a). Small differences in the depolarizing responses to ACh and nicotine of oocytes expressing several different subunit combinations suggest that the members of this receptor family may also differ in their responses to different agonists.

Multiple functional subtypes of neuronal nAChRs are present in the nervous system, as recent biophysical and pharmacological studies have demonstrated (for reviews, see Steinbach and Ifune, 1989; Luetje et al., 1990b), and the expression of different functional subtypes is developmentally regulated (Schuetze and Role, 1987; Moss et al., 1989). The large number of functional neuronal nAChRs that can be formed in oocytes suggests that differential pairing of α- and β-subunits may be a mechanism by which multiple subtypes of neuronal nAChRs are generated in the nervous system. To address this issue, we examined the agonist sensitivity of 6 functional α–β-subunit combinations that can be formed with α2, α3, α4, β2, and β4. We demonstrate that each subunit combination displays a distinct pattern of sensitivity to 4 nicotinic agonists. We conclude that both the α- and the β-subunits contribute to the pharmacological character of neuronal nAChRs.

Materials and Methods

Materials. Xenopus laevis frogs were purchased from Nasco (Fort Atkinson, WI). RNA transcription kits were from Stratagene (La Jolla, CA). Diguanosine triphosphate was from Pharmacia (Piscataway, NJ). SP6 polymerase and RNasin were from Promega (Madison, WI). Acetylcholine, (−)-nicotine, cytisine, 1,1-dimethyl-4-phenylpiperazinium (DMPP), atropine, 3-aminobenzoic acid ethyl ester, and collagenase type I were from Sigma (St. Louis, MO). Neuronal bungarotoxin (toxin

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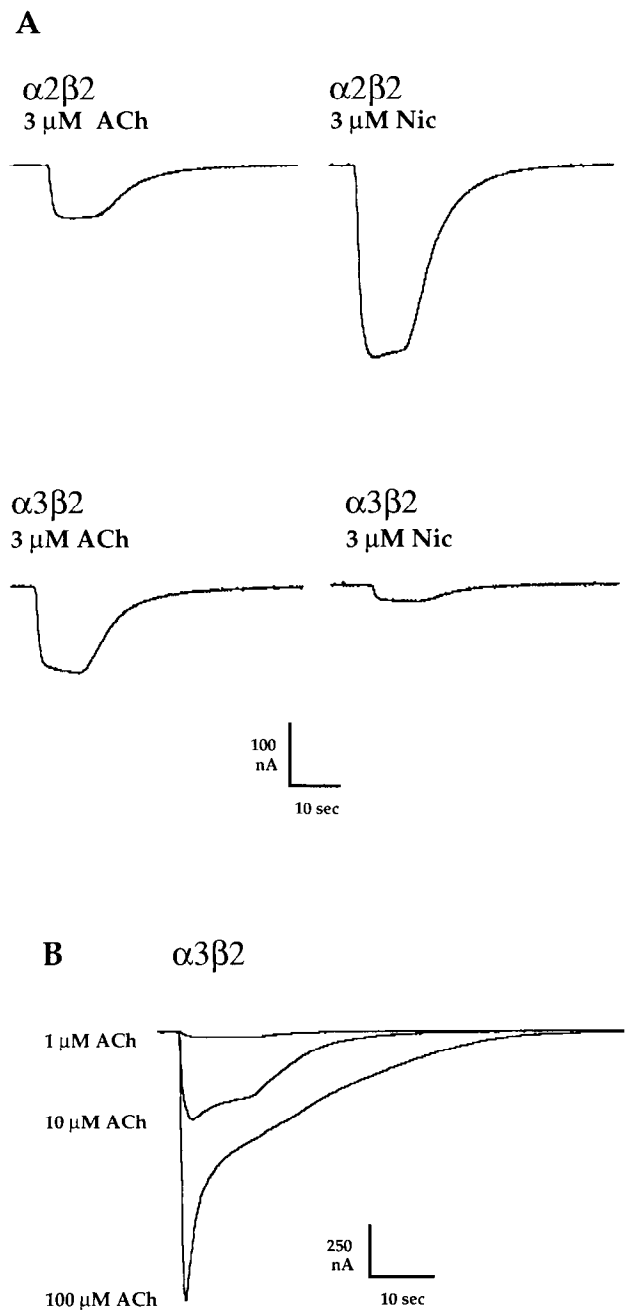

Figure 1. Current responses of oocytes expressing neuronal nAChR subunit combinations. *A*, The $\alpha2\beta2$ and $\alpha3\beta2$ subunit combinations differ in their responses to ACh and nicotine (*Nic*). Current responses of an $\alpha2\beta2$-expressing oocyte and an $\alpha3\beta2$-expressing oocyte elicited by ACh and nicotine were measured under 2-electrode voltage clamp. Each agonist was applied at a concentration of 3 μM. Oocytes were held at −70 mV. *B*, High agonist concentrations cause rapid, extensive desensitization. Current responses of an $\alpha3\beta2$-expressing oocyte in response to 1, 10, and 100 μM ACh were measured under 2-electrode voltage clamp at a holding potential of −70 mV.

F) was a gift from Dr. Richard Zigmond (Department of Neurosciences, Case Western Reserve University, Cleveland, OH).

Injection of in vitro *synthesized RNA into* Xenopus *oocytes.* Diguanosine triphosphate-capped RNA was synthesized *in vitro* from linearized template DNA encoding the $\alpha1$, $\alpha2$, $\alpha3$, $\alpha4$, $\beta1$, $\beta2$, $\beta4$, γ-, and δ-subunits as previously described (Boulter et al., 1987) using an RNA transcription kit, RNasin, and SP6 polymerase. Mature *Xenopus laevis* were anesthetized by submersion in 0.1% 3-aminobenzoic acid ethyl ester, and oocytes were then surgically removed. Follicle cells were removed by treatment with collagenase type I for 2 hr at room temperature. Each oocyte was injected with a total of 2.5–10 ng RNA in 50 nl water and incubated at 19°C in Barth's saline for 2–7 d (Coleman, 1984). The ratio of RNA encoding each subunit was 1:1 unless otherwise noted.

Electrophysiological recordings. Oocytes were perfused at room temperature (20–25°C) in a 300-μl chamber with a solution containing 115 mM NaCl, 1.8 mM $CaCl_2$, 2.5 mM KCl, 10 mM HEPES (pH, 7.2), and 1 μM atropine (perfusion solution). Perfusion was continuous at a rate of approximately 10 ml/min. Agonists were diluted in perfusion solution and applied using a solenoid valve to switch from perfusion solution to agonist solution. The oocytes were exposed to agonist for approximately 10 sec. Current responses to agonist application were studied under 2-electrode voltage clamp at a holding potential of −70 mV except where noted. Experiments were performed using an Axoclamp 2A voltage-clamp unit (Axon Instruments, Burlingame, CA). Micropipettes were filled with 3 M KCl and had resistances of 0.5–3.0 MΩ. Agonist-induced responses were filtered at 15 Hz (8-pole Bessel, Frequency Devices, Haverhill, MA), then captured, stored, and analyzed using Labview software (National Instruments, Austin, TX) and a Macintosh II computer.

Extensive desensitization at high agonist concentrations makes full dose–response curves misleading. We therefore constructed partial dose–response curves; the agonist-induced responses of each oocyte were normalized with respect to the response of the same oocyte to 1 μM acetylcholine (neuronal nAChR in Figs. 2,4) or 30 nM acetylcholine (muscle nAChR in Fig. 3). One micromolar acetylcholine was chosen as the standard when working with neuronal nAChR because this concentration was low enough that little desensitization occurred and thus reproducible responses to the standard could be obtained over the course of the experiment. One micromolar acetylcholine was also high enough that oocytes expressing each of the different receptor subunit combinations responded reliably. Oocytes were washed for approximately 5 min between applications of concentrations of agonists causing little desensitization. Responses to higher, desensitizing concentrations of agonists were usually obtained at the end of experiments. Dose–response data were fit by a nonlinear least-squares program using the equation

$$\text{current} = (\text{maximum current})/[1 + (EC_{50}/[\text{agonist}])^n],$$

where n and EC_{50} represent the Hill coefficient and the agonist concentration producing half-maximal response, respectively.

Results

Each neuronal nAChR subunit combination has unique properties

Our investigation of the agonist pharmacology of neuronal nAChRs revealed striking differences between receptors of differing subunit composition. An example is shown in Figure 1*A*. Oocytes expressing neuronal nAChRs of the $\alpha2\beta2$ or $\alpha3\beta2$ subtype were tested for responses to bath-applied ACh and nicotine under 2-electrode voltage clamp. The current elicited in an $\alpha2\beta2$-expressing oocyte by 3 μM nicotine was more than 3-fold larger than the current elicited by an equal concentration of ACh. In sharp contrast, the current elicited in an $\alpha3\beta2$-expressing oocyte by 3 μM nicotine was approximately 5-fold smaller than the current elicited by an equal concentration of ACh. The 15-fold difference in the ratio of these responses demonstrates pharmacological diversity among neuronal nAChRs composed of different subunit combinations.

Because rapid, extensive desensitization of nAChRs occurs at high agonist concentrations (Fig. 1*B*), and because the size and shape of *Xenopus* oocytes make rapid application of agonists difficult, determination of the true peak response to high concentrations of agonist is problematic. The maximal response is therefore an unreliable standard with which to normalize data. In the absence of complete dose–response curves (and consequent EC_{50} estimations), a comparison of the effect of a single agonist upon oocytes expressing different subunit combinations is not possible. Differences in the magnitude of the responses

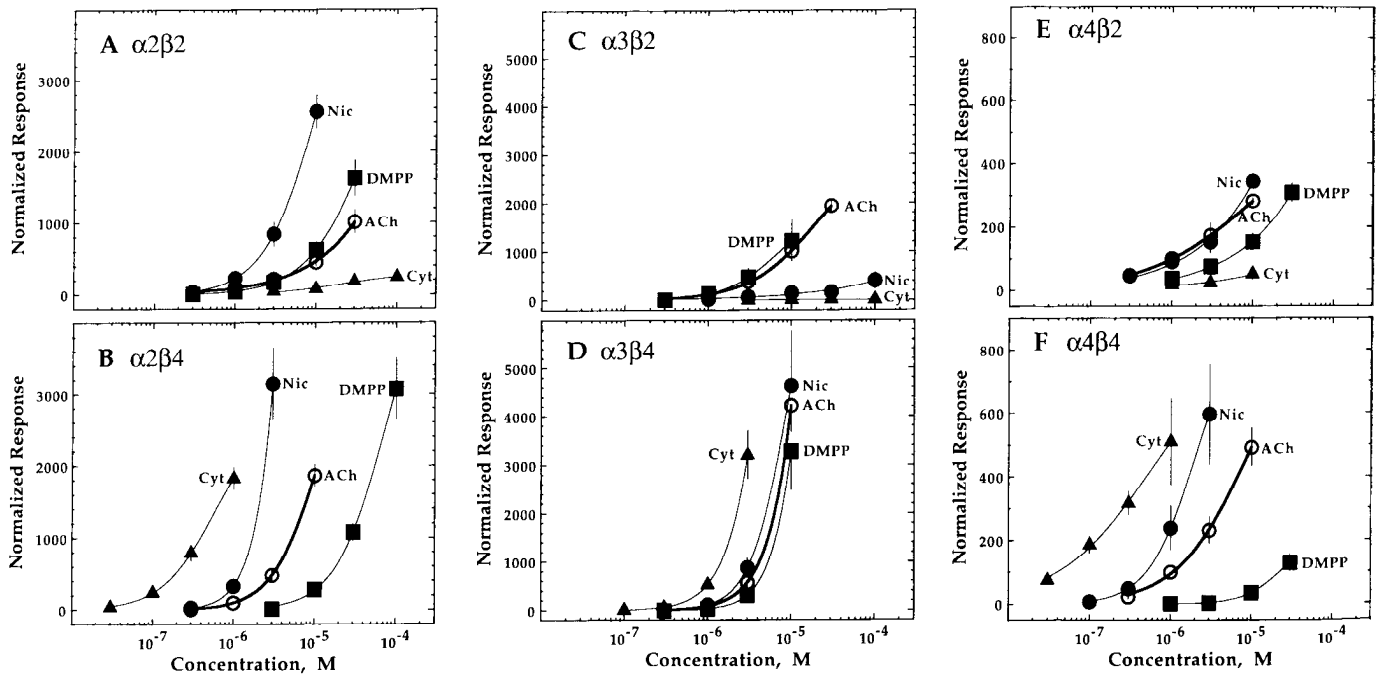

Figure 2. Each neuronal nAChR subunit combination has unique pharmacological properties. Agonist-induced current responses of oocytes expressing $\alpha2\beta2$ (*A*), $\alpha2\beta4$ (*B*), $\alpha3\beta2$ (*C*), $\alpha3\beta4$ (*D*), $\alpha4\beta2$ (*E*), or $\alpha4\beta4$ (*F*) were measured under 2-electrode voltage clamp at a holding potential of −70 mV. Currents were elicited by bath application of various concentrations of *ACh* (*open circles*), nicotine (*Nic*; *solid circles*), cytisine (*Cyt*; *triangles*), and *DMPP* (*squares*). Responses are presented after normalization to the response of the same oocyte to 1 μM ACh (response to 1 μM ACh = 100). Each *point* represents the mean ± SD of the responses of 3–8 separate oocytes.

of 2 different receptor-expressing oocytes to a single agonist could easily be due to the extreme variability in the extent to which oocytes express injected RNA (Tate et al., 1990). Thus, in order to explore the pharmacological differences among neuronal nAChRs, we constructed partial dose–response curves for each subunit combination in response to a panel of 4 different agonists and normalized all data relative to the response to 1 μM ACh (Fig. 2). Specifically, we used the agonists ACh, nicotine, cytisine, and DMPP to characterize the 6 pairwise α–β-subunit combinations that can be formed with the α2, α3, α4, β2, and β4 subunits. These results were obtained using 2–3 different lots of RNA and oocytes from 2–6 different donors.

Structural homology with the α-subunit of muscle and *Torpedo* nAChRs suggests that the α-subunits of neuronal nAChRs contain the agonist binding site. For this reason, the α-subunits would be expected to contribute to the pharmacologic character of neuronal nAChRs. Results presented in Figures 1 and 2 confirm this expectation. Oocytes expressing the $\alpha2\beta2$ subunit combination show greater sensitivity to nicotine (Fig. 2*A*, solid circles) than to ACh (open circles). In contrast, oocytes expressing the $\alpha3\beta2$ subunit combination are much less sensitive to nicotine than to ACh (Fig. 2*C*). Oocytes expressing the $\alpha4\beta2$ subunit combination show equal sensitivity to both agonists (Fig. 2*E*). Clearly then, because each of these receptors includes the same β-subunit (β2), the different α-subunits contribute to the differences in sensitivity to nicotine and ACh.

A contribution by the α-subunit to the pharmacological character of neuronal nAChRs is not surprising, given the probable location of the agonist binding site on the α-subunit. However, the results presented in Figure 2 show that the β-subunit also contributes to the pharmacological character of neuronal nAChRs. Receptors that include the β2 subunit ($\alpha2\beta2$, $\alpha3\beta2$, and $\alpha4\beta2$) are almost completely unresponsive to cytisine (Fig. 2*A,C,E*, triangles). In contrast, receptors that contain the β4 subunit ($\alpha2\beta4$, $\alpha3\beta4$, and $\alpha4\beta4$) are more sensitive to cytisine than to any other agonist tested (Fig. 2*B,D,F*). Thus, differences in sensitivity to cytisine do not appear to be a function of which presumed agonist-binding subunit (α2, α3, or α4) is present; rather they appear to be a function of the particular β-subunit (β2 or β4) present in the receptor.

Use of DMPP (Fig. 2, squares) also reveals a contribution by the β-subunit to the pharmacologic character of neuronal nAChRs. Receptors containing α3 with either β2 or β4 are equally sensitive to ACh and DMPP (Fig. 2*C,D*). While the $\alpha2\beta2$ subtype was also equally sensitive to both ACh and DMPP (Fig. 2*A*), the $\alpha2\beta4$ subtype was about 5-fold more sensitive to ACh than to DMPP (Fig. 2*B*). Receptors containing α4 with either β2 or β4 were more sensitive to ACh than to DMPP. However, the respective differences in sensitivity were about 4-fold for $\alpha4\beta2$ and approximately 24-fold for $\alpha4\beta4$ (Fig. 2*E,F*). The differences between the $\alpha2\beta2$ and $\alpha2\beta4$ subtypes and between the $\alpha4\beta2$ and $\alpha4\beta4$ subtypes demonstrate the importance of the β-subunit in determining the relative sensitivity to DMPP and ACh.

The importance of both α- and β-subunits in defining the pharmacologic properties of neuronal nAChRs can be illustrated by a comparison of the rank order of potency of these agonists for each receptor (Table 1). If the receptor contains the β2 subunit, then the rank order of potency is unique to the particular α-subunit present. However, when each α-subunit forms a receptor with β4, the rank order of potency differs dramatically from that of the corresponding β2-containing receptor. Although the rank orders of potency for all the β4-containing receptors are similar, differences can be seen in a comparison of the sen-

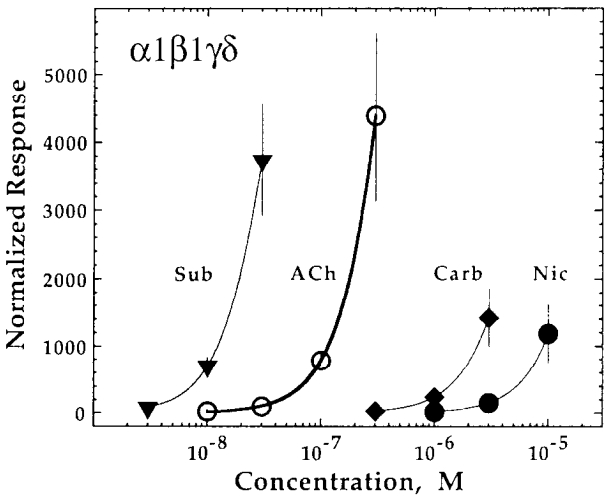

Figure 3. Current responses of $\alpha 1\beta 1\gamma\delta$-expressing oocytes. Agonist-induced current responses of $\alpha 1\beta 1\gamma\delta$-expressing oocytes were measured under 2-electrode voltage clamp at a holding potential of −70 mV. Currents were elicited by bath application of various concentrations of *ACh* (*open circles*), nicotine (*Nic*; *solid circles*), suberyldicholine (*Sub*; *triangles*), and carbachol (*Carb*; *diamonds*). Each response has been normalized to the response of the same oocyte to 30 nM ACh (response to 30 nM ACh = 100). Each *point* represents the mean + SD of the responses of 3 separate oocytes.

sitivities of each subunit combination to the most potent and least potent of the agonists (cytisine and DMPP, respectively). For $\alpha 3\beta 4$, the difference is approximately 3-fold; for $\alpha 2\beta 4$, the difference is approximately 70-fold; and for $\alpha 4\beta 4$, the difference is approximately 400-fold. Thus, both α- and β-subunits contribute to the pharmacologic character of neuronal nAChRs.

Oocytes express nAChRs accurately

The results presented above demonstrate that different combinations of α- and β-subunits produce receptors that display distinct pharmacological properties. These results may reflect characteristics that these receptor subunit combinations possess *in vivo*. However, alternative explanations are possible. Of major importance is the question of whether characteristics of ligand-gated ion channels expressed in the oocyte system accurately represent *in vivo* function. Could the diversity seen with neuronal nAChRs expressed in oocytes have more to do with the oocyte than with the characteristics of neuronal nAChRs *in vivo*? Therefore, it is important to demonstrate the fidelity of the oocyte expression system with respect to the functional characteristics under study.

To determine whether the pharmacological characteristics of ligand-gated ion channels are accurately expressed in oocytes, we tested the mouse muscle nAChR (Fig. 3). This receptor, of subunit composition $(\alpha 1)_2\beta 1\gamma\delta$, is expressed by the mouse muscle cell line BC3H-1, where responses to agonists have been characterized in detail (Sine and Steinbach, 1986, 1987). The cDNA templates from which $\alpha 1$, $\beta 1$, γ-, and δ-RNA were transcribed were isolated from a BC3H-1 cDNA library, offering an excellent opportunity to compare pharmacological characteristics of a receptor expressed both *in vivo* and in the *Xenopus* oocyte. Activation of nAChRs on BC3H-1 cells occurs with a rank order of potency of suberyldicholine > ACh > carbachol (Sine and Steinbach, 1986, 1987). We found that the rank order of potency for these agonists was identical for oocytes expressing the mouse muscle nAChR (Fig. 3). Moreover, the ratio of equally effective concentrations of suberyldicholine, ACh, and carbachol were quite similar in both systems: 0.5:1.0:20.0, respectively, in BC3H-1 cells and 0.12:1.0:20.3, respectively, in oocytes. We also found that mouse muscle nAChR-expressing oocytes were approximately 50–100-fold less responsive to nicotine than to ACh.

Another alternative explanation of our results is that differences in subunit availability, rather than different subunit combinations, underlie the pharmacological differences we have observed. RNAs encoding different subunits might be expressed to differing extents in the oocyte, leading to differential availability of subunits and resulting in receptors of differing subunit stoichiometry. We controlled for this possibility in 2 ways: First, all pharmacological differences presented in Figure 2 were obtained with 2–3 different lots of RNA encoding each receptor subunit, as well as with oocytes from 2–6 different donors. This rules out the possibility that the differences presented in Figure 2 artifactually result from the use of an unusual lot of RNA or set of oocytes. Second, we examined the effect of altering the ratio of α- to β-subunit RNA on the pharmacological properties of the expressed receptors (Fig. 4). Oocytes were injected with RNA encoding the $\alpha 4$ and $\beta 4$ subunits at ratios of 1:4 (Fig. 4*A*), 1:1 (Fig. 4*B*), and 4:1 (Fig. 4*C*). We found that, in each case, the agonist rank order of potency for the expressed receptors was identical. The same experiment, which spans a 16-fold difference in RNA ratio, was performed with the $\alpha 3\beta 2$ subunit combination, with a similar lack of effect on the agonist pharmacology of the expressed receptor (data not shown). These results demonstrate that differences in subunit availability do not underlie the pharmacological differences presented above.

Table 1. Pharmacological properties of neuronal nAChR subunit combinations

Subunit combination	Equally effective concentrations of agonist relative to ACh				
	ACh	Nic	Cyt	DMPP	Rank order of potency
$\alpha 2\beta 2$	1	0.2	14	1	Nic > DMPP = ACh > Cyt
$\alpha 2\beta 4$	1	0.4	0.07	5	Cyt > Nic > ACh > DMPP
$\alpha 3\beta 2$	1	17	>100	1	DMPP = ACh > Nic > Cyt
$\alpha 3\beta 4$	1	1	0.4	1	Cyt > Nic = ACh = DMPP
$\alpha 4\beta 2$	1	1	>30	4	Nic = ACh > DMPP > Cyt
$\alpha 4\beta 4$	1	0.3	0.06	24	Cyt > Nic > ACh > DMPP

Equally effective concentrations and rank orders of potency are derived from the data presented in Figure 2. Cyt, cytisine; Nic, nicotine.

Recently, Hartman and Claudio (1990) reported the presence of RNA encoding a muscle nAChR α-subunit in *Xenopus* oocytes, while Buller and White (1990) reported expression of functional muscle nAChRs in oocytes following injection of RNA encoding *Torpedo* β-, γ-, and δ-subunits. To determine whether the potential presence of endogenous nAChRs might affect the interpretation of our results, we attempted to detect the presence of any endogenous nAChRs in 2 different types of experiments. First, oocytes sham injected with water were tested for current responses to ACh, nicotine, cytisine, and DMPP, each at a concentration of 100 μM. No responses were observed. Second, to test for the presence of a muscle α-subunit in the absence of β-, γ-, or δ-subunits, we injected oocytes with RNA encoding the muscle β-, γ-, and δ-subunits and tested these oocytes for current responses to 100 μM each of ACh, suberyldicholine, and nicotine. Again, no responses were observed. Also, oocytes injected with the neuronal $\beta 2$ subunit alone were unresponsive to 100 μM ACh. The lack of evidence for endogenous nAChRs in our experiments is not surprising because the responses reported by Buller and White (1990) only exceeded 1 nA when the concentration of applied ACh was 100 μM or greater, while none of our experiments involved ACh concentrations greater than 100 μM. Also, the *Xenopus* muscle nAChR α-subunit may form functional receptors less efficiently with mouse muscle β-, γ-, and δ-subunits than with *Torpedo* β-, γ-, and δ-subunits.

Differential effects of nicotine and cytisine result from different mechanisms

The results presented in Figures 1 and 2 suggest that nicotine and cytisine are good probes of the structure–function relationships of neuronal nAChRs. Nicotine is a weak agonist on $\alpha 3\beta 2$, but a potent agonist on $\alpha 2\beta 2$ and $\alpha 4\beta 2$, providing insight into the functional contribution of the α-subunits. Cytisine is a weak agonist on $\beta 2$-containing receptors, but becomes a potent agonist by inclusion of the $\beta 4$ subunit rather than the $\beta 2$ subunit, clearly demonstrating a functional contribution by the β-subunits. There are several potential explanations for these observations. Weak responses may simply reflect a low affinity of receptor for agonist. Alternatively, the receptor might have a high affinity for agonist, but the response is blocked through some subsequent effect of the agonist. Another possibility is that cytisine or nicotine might be partial agonists, in which case, occupation of the agonist binding site would not as efficiently result in open ion channels (Colquhoun et al., 1987). To determine which of these possibilities best explains our observations with nicotine and cytisine, we compared the response of $\alpha 3\beta 2$-expressing oocytes to 3 μM ACh in the absence and presence of various concentrations of nicotine or cytisine (Fig. 5*A*). If the weak responses to nicotine or cytisine are due to low affinity of the receptor for these agonists, then the presence of nicotine or cytisine should have little effect on the response to ACh. If the weak responses are due to partial agonism, or to a secondary blocking effect of nicotine or cytisine, then the ACh response should be blocked when ACh is applied in the presence of a sufficiently high concentration of nicotine or cytisine.

The response to 3 μM ACh was not significantly affected by coapplication of nicotine (Fig. 5A, circles) at concentrations ranging from 100 nM to 10 μM. This suggests that the weak response of $\alpha 3\beta 2$-expressing oocytes to nicotine is a consequence of a low affinity of this subunit combination for nicotine. In contrast, coapplication of cytisine (Fig. 5*A*, triangles) results in dramatic inhibition of the response to 3 μM ACh, with an IC_{50} for cytisine of approximately 400 nM. This result suggests that the weak responses of $\beta 2$-containing receptors to cytisine result from a blocking effect of cytisine secondary to its role as an

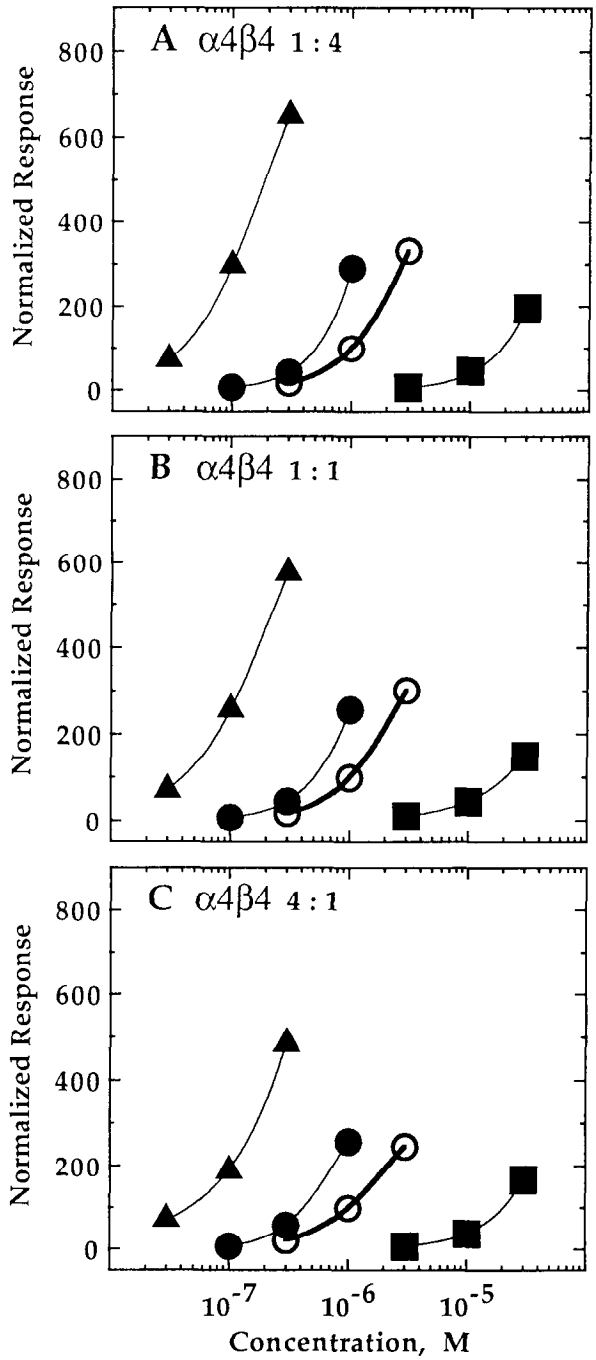

Figure 4. Alteration of the ratio of $\alpha 4$ and $\beta 4$ subunit RNA does not affect the pharmacology of the expressed receptors. Oocytes were injected with RNA encoding the $\alpha 4\beta 4$ subunit combination as in Figure 2, except that the ratio of α-RNA to β-RNA injected into each oocyte was 1:4 (*A*), 1:1 (*B*), or 4:1 (*C*). Agonist-induced current responses were measured under 2-electrode voltage clamp at a holding potential of −70 mV. Currents were elicited by bath application of various concentrations of ACh (*open circles*), nicotine (*solid circles*), cytisine (*triangles*), and DMPP (*squares*). Each response was normalized to the response of the same oocyte to 1 μM ACh (response to 1 μM ACh = 100). Each *point* represents the mean of the responses of 2–3 separate oocytes, which usually varied from the mean by 15% or less.

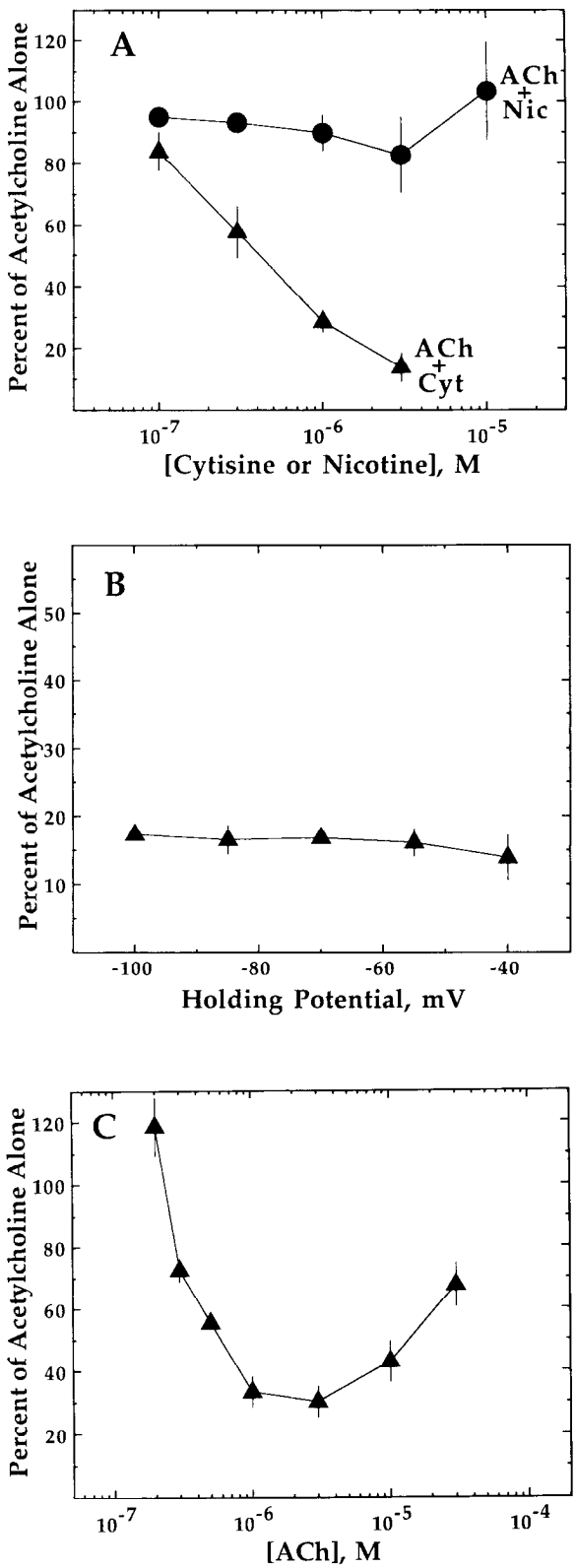

Figure 5. Examination of the weak response of $\alpha3\beta2$-expressing oocytes to nicotine (*Nic*) and cytisine (*Cyt*). *A*, Low concentrations of cytisine, but not nicotine, block the response of $\alpha3\beta2$-expressing oocytes to ACh. Agonist-induced current responses of $\alpha3\beta2$-expressing oocytes were measured under 2-electrode voltage clamp at a holding potential of −70 mV. Current responses were elicited by bath application of 3 μM ACh in the absence and presence of various concentrations of nicotine (*circles*) or cytisine (*triangles*). Responses to ACh in the presence of nicotine or cytisine are presented as a percent of the response to ACh alone. Each *point* represents the mean ± SD of the responses of 3–4 separate oocytes. *B*, Cytisine blockade of the response of $\alpha3\beta2$-expressing oocytes to ACh is not voltage dependent. Current responses of $\alpha3\beta2$-expressing oocytes elicited by 3 μM ACh in the absence and presence of 3 μM cytisine were measured under 2-electrode voltage clamp at a variety of holding potentials. Responses to ACh in the presence of cytisine are presented as a percent of the response to ACh alone. Each *point* represents the mean ± SD of the responses of 3 separate oocytes. *C*, Cytisine blockade of the response of $\alpha3\beta2$-expressing oocytes to ACh is dependent upon the concentration of ACh. Current responses of $\alpha3\beta2$-expressing oocytes elicited by various concentrations of ACh in the absence and presence of 1 μM cytisine were measured under 2-electrode voltage clamp at a holding potential of −70 mV. Responses to ACh in the presence of cytisine are presented as a percent of the response to ACh alone. Each *point* represents the mean ± SD of the responses of 3–9 separate oocytes.

agonist, or from cytisine being a partial agonist. Possible mechanisms that might account for a blocking effect of cytisine include open-channel block (Sine and Steinbach, 1984) or an effect of cytisine at an allosteric modulatory site (Changeux et al., 1987).

Open-channel blockade of nAChRs is often voltage dependent (Sine and Steinbach, 1984). To determine if the blocking effect of cytisine is voltage dependent, we examined the blockade of $\alpha3\beta2$ activation by 3 μM ACh in the presence of 3 μM cytisine at various holding potentials. Figure 5*B* shows that cytisine blockade of ACh responses is not voltage dependent at holding potentials ranging from −100 to −40 mV.

Blockade by open-channel blockers and competitive ligands such as partial agonists can show a dependence upon the concentration of agonist (in this case, ACh). With a preference for the open form of the channel, open-channel blocking agents cause an increasing degree of blockade as the concentration of agonist, and hence the proportion of open channels, increases. In contrast, apparent blockade by a partial agonist should decrease as increasing concentrations of full agonist (ACh) outcompete the partial agonist for the binding site. To determine whether cytisine blockade of ACh responses is dependent upon the concentration of ACh, we examined the extent to which responses of $\alpha3\beta2$-expressing oocytes to various concentrations of ACh were blocked by 1 μM cytisine (Fig. 5*C*). As the concentration of ACh increased from 200 nM to 1 μM, cytisine showed an increasing ability to block the response. This result suggests that, in its blocking activity, cytisine has a preference for the open form of the receptor and might then be an open-channel blocker. However, as the concentration of ACh was increased from 3 to 30 μM, cytisine showed a decreasing ability to block the response to ACh. This suggests that, in this concentration range, ACh is competing with cytisine for the site at which cytisine exerts its inhibitory effect. The apparent enhancement of the 200 nM ACh response by cytisine can be easily accounted for. At 200 nM, the ACh responses are small (3.4 ± 0.3 nA). It should be remembered that cytisine is an agonist and does elicit small current responses from $\alpha3\beta2$-expressing oocytes (approximately 0.5 nA at 1 μM cytisine). Thus, in the absence of blocking activity by cytisine, the responses to ACh and cytisine are additive, and the sum is greater than the response to ACh alone.

Our results suggest that the near unresponsiveness of receptors containing the $\beta2$ subunit to cytisine is due to a blocking effect of cytisine separate from its role as an agonist. Cytisine is not a partial agonist because, as a partial agonist, cytisine would

show only a decreasing ability to block as the ACh concentration increased. Cytisine may be a voltage-independent open-channel blocker active at very low concentrations. Although open-channel blockade of muscle and neuronal nAChRs by ACh has only been shown to occur at very high agonist concentrations (>300 μM ACh; Sine and Steinbach, 1984; Oortgiesen and Vijverberg, 1989), suberyldicholine has been shown to cause open-channel block of muscle nAChRs with an equilibrium dissociation constant of about 6 μM (Ogden and Colquhoun, 1985). Alternatively, cytisine may be acting at an inhibitory allosteric modulatory site on the receptor. Although we have not, at present, distinguished between these 2 alternatives, it is clear that the sensitivity of neuronal nAChRs to cytisine is a function of the particular β-subunit present in the receptor.

Discussion

α- and β-subunits each make contributions to the agonist-binding properties of neuronal nAChR

In this study, we investigated the agonist pharmacology of neuronal nAChRs composed of various combinations of α- and β-subunits. We demonstrate that each neuronal nAChR subunit combination displays an unique set of pharmacological properties. We provide evidence that these pharmacological differences are not an artifactual result of using the *Xenopus* oocyte expression system, and we conclude that the diverse pharmacological properties of these neuronal nAChRs are due to contributions by both the α- and the β-subunits present in each receptor subtype.

The results presented in Figure 2 reveal a number of examples of diversity among neuronal nAChRs composed of different combinations of subunits. This diversity cannot be explained only as a consequence of the particular α-subunit present in a particular subunit combination. It is clear that the particular β-subunit present also makes a contribution to the pharmacological character of the receptor. This can be appreciated most fully by considering the α3β2 subunit combination in detail. The α3β2 receptor differs from the other 5 subunit combinations examined by being relatively insensitive to nicotine. Furthermore, the results presented in Figure 5*A* suggest that the α3β2 subunit combination is much less sensitive to nicotine than to ACh as a result of a low affinity for nicotine.

The response of α3β2 to nicotine differs from the responses of other β2-containing receptors (α2β2 and α4β2). The nicotine sensitivity of α3β2 also differs from that of the other α3-containing receptor (α3β4). Thus, receptors are more sensitive to nicotine when they are formed by the β2 subunit and a different α-subunit (α2 or α4), or when they are formed by the α3 subunit and a different β-subunit (β4), than when α3 and β2 form a receptor together. This is strong evidence that both α- and β-subunits contribute to the pharmacological character of neuronal nAChRs.

The α3β2 subunit combination is similar to other β2-containing subunit combinations in being almost unresponsive to cytisine, while in contrast, α3β4 and the other β4-containing subunit combinations are highly sensitive to cytisine. Thus, it is the β-subunit that is primarily responsible for determining the sensitivity of neuronal nAChRs to cytisine. The data presented in Figure 5 suggest that the weak responses to cytisine are not due to partial agonism, but, rather, to a blocking activity of cytisine secondary to its role as an agonist. This blocking activity may be due to cytisine acting as an open-channel blocker or at an inhibitory allosteric modulatory site.

A contribution by the β-subunit to the pharmacological character of neuronal nAChRs has also been reported by Duvoisin et al. (1989). In this study, nearly complete functional blockade of the α3β2 subtype was achieved by incubation with 100 nM neuronal bungarotoxin (also known as bungarotoxin 3.1, toxin F, and K-bungarotoxin), but the α3β4 subtype was insensitive to this concentration of neuronal bungarotoxin. We have repeated this experiment and obtained similar results: Incubation with 100 nM neuronal bungarotoxin, which results in complete functional blockade of the α3β2 subtype (Luetje et al., 1990b; data not shown), failed to achieve blockade of the α3β4 subtype ($n = 3$).

The contributions of an α-subunit to the pharmacologic properties of nAChRs are not difficult to envision, because the α-subunit is the likely location of the agonist binding site. Contributions by the β-subunit may be more complex. There are several possible mechanisms by which the β-subunit might contribute to the pharmacologic character of neuronal nAChRs. Although the agonist binding sites of nAChRs are thought to be primarily on the α-subunits (Kao et al., 1984; Dennis et al., 1988), this does not rule out the direct physical participation of the β-subunit in formation of the agonist binding site with the α-subunit. Another possibility is that the β-subunit might act indirectly through its association with the α-subunit to alter the agonist binding site, even though this site might be located entirely on the α-subunit. The β-subunit might also provide or modify an allosteric modulatory site or channel blocking site at which a ligand could act. Figure 5*A* suggests that the weak response of α3β2 to nicotine is due to a low affinity of this receptor for this agonist. This implies that the β-subunit is influencing the properties of the agonist binding site. Figure 5*A–C* suggests that the weak responses of β2-containing receptors to cytisine, as compared to β4-containing receptors, are due to the action of cytisine as an open-channel blocker or at an allosteric modulatory site. In this case, the β-subunit may be either providing or altering this site.

There have been several demonstrations that non-α-subunits can contribute to the functional properties of ligand-gated ion channels. The nAChRs of the *Torpedo* electric organ and the BC3H-1 mouse muscle cell line possess 2 nonequivalent ligand binding sites (Neubig and Cohen, 1979; Sine and Taylor, 1980, 1981). This appears to result from the fact that one binding site is formed by an α-subunit associated with a γ-subunit, while the other binding site is formed by an α-subunit associated with a δ-subunit (Blount and Merlie, 1989; Pedersen and Cohen, 1990). The properties of $GABA_A$ receptors have also been shown to be modified by non-α-subunits. Although functional $GABA_A$ receptors can be formed by pairwise combination of α- and β-subunits, the presence of the γ2 subunit is necessary for these receptors to display benzodiazepine sensitivity (Pritchett et al., 1989).

Pharmacological properties of nAChRs expressed in oocytes accurately reflect in vivo *properties*

The validity of our conclusions about pharmacological diversity among neuronal nAChRs depends upon several factors. One is the functional accuracy with which exogenous proteins are expressed by the oocyte. To examine this issue with respect to the pharmacological properties under study, we compared the characteristics of the mouse muscle nAChR expressed in oocytes with those reported for the native mouse muscle nAChR expressed in the BC3H-1 cell line (Sine and Steinbach, 1986, 1987).

We found the characteristics of the receptor expressed in oocytes to be quite similar to those of the native receptor in BC3H-1 cells. This shows that, at least for pharmacological studies, the oocyte can express nAChRs accurately. There are a number of examples of other properties of ligand- and voltage-gated ion channels that are expressed accurately in *Xenopus* oocytes. For example, the single-channel properties of bovine $\alpha 1\beta 1\gamma\delta$ and $\alpha 1\beta 1\epsilon\delta$ expressed in oocytes have been shown to be quite similar to the single-channel properties of nAChRs expressed in fetal and adult bovine muscle, respectively (Mishina et al., 1986). Also, Koren et al. (1990) found that a cloned K^+ channel displayed identical whole-cell current properties whether expressed in oocytes or in a mammalian myoblast cell line.

The accuracy of our observations might also be affected by differences in the degree of expression of different RNA transcripts, perhaps leading to artificial variation of subunit stoichiometry. This might then be more important in accounting for the pharmacological differences we have characterized than differences in subunit combination. We have ruled out this possibility by using multiple lots of both RNA and oocytes and by showing that varying the ratio of α- to β-RNA injected into the oocytes does not alter the pharmacological properties of the expressed receptors (Fig. 4). Papke et al. (1989a) presented evidence indicating that alteration of the ratio of α- to β-subunit RNA injected into oocytes could favor the expression of 1 of 2 single-channel conductance states. Although differential availability of α- and β-subunits may have an effect upon single-channel conductance, we have shown that pharmacological properties of the expressed receptors are not affected.

The results we have presented are consistent with several earlier studies characterizing various neuronal nAChR subunit combinations. Voltage–response recordings have shown rat $\alpha 3\beta 2$ expressed in oocytes to be somewhat more sensitive to ACh than to nicotine (Boulter et al., 1987). Similar experiments have also shown rat $\alpha 3\beta 4$ to be approximately equally sensitive to both ACh and nicotine, while rat $\alpha 2\beta 4$ was more sensitive to nicotine than to ACh (Duvoisin et al., 1989). A voltage-clamp study of avian $\alpha 4\beta 2$ (Bertrand et al., 1990) described agonist sensitivities quite similar to those we have found with the rat $\alpha 4\beta 2$.

Differential subunit assembly can generate receptor diversity

A common feature of ligand-gated ion channel families, such as those gated by ACh and GABA, is the large number of homologous subunits that have been identified. The numerous functionally distinct subunit combinations that can be formed in oocytes suggest differential subunit association as a mechanism by which a large number of structurally and functionally distinct receptors can be generated *in vivo*. In fact, there have been a number of studies demonstrating biophysical and pharmacological diversity among neuronal nAChRs (for reviews, see Steinbach and Ifune, 1989; Luetje et al., 1990b), as well as developmental regulation of the expression of different functional subtypes (Schuetze and Role, 1987; Moss et al., 1989). Understanding this diversity is important when considering processes involving nAChRs in the nervous system. For example, the presence of multiple, functionally diverse nAChRs in the nervous system may have relevance to understanding the psychoactive and addictive properties of nicotine. Nicotine addiction may involve all or only a subset of the nAChR subtypes present in the nervous system.

Pharmacological definition of neuronal nAChRs expressed in oocytes will aid in the identification of nAChR subtypes *in vivo*. For example, neuronal bungarotoxin is able to distinguish $\alpha 3\beta 2$, which is highly sensitive to blockade by this toxin, from subunit combinations showing little ($\alpha 4\beta 2$) or no sensitivity to this toxin (Duvoisin et al., 1989; Luetje et al., 1990a). Cytisine can distinguish between receptors containing $\beta 4$, which are highly sensitive to this agonist, and receptors containing $\beta 2$, which are almost completely insensitive to this agonist (Fig. 2). The ratio of equally effective concentrations of nicotine and ACh can serve as a criterion for distinguishing between $\alpha 2\beta 2$ (nicotine/ACh = 0.2), $\alpha 3\beta 2$ (nicotine/ACh = 17), and $\alpha 4\beta 2$ (nicotine/ACh = 1). Also useful for differentiating between these subunit combinations is the antagonist dihydro-β-erythroidine (Luetje and Patrick, 1989). The ratio of equally effective concentrations of DMPP and ACh can be employed to distinguish between $\alpha 2\beta 4$ (DMPP/ACh = 5), $\alpha 3\beta 4$ (DMPP/ACh = 1), and $\alpha 4\beta 4$ (DMPP/ACh = 24). In addition to serving as criteria for identifying receptor subtypes *in vivo*, the distinct pharmacological differences between these receptors will provide a foundation for the study of structure–function relationships of neuronal nAChRs.

References

Bertrand D, Ballivet M, Rungger D (1990) Activation and blocking of neuronal nicotinic acetylcholine receptor reconstituted in *Xenopus* oocytes. Proc Natl Acad Sci USA 87:1993–1997.

Blount P, Merlie JP (1989) Molecular basis of the two nonequivalent ligand binding sites of the muscle nicotinic acetylcholine receptor. Neuron 3:349–357.

Boulter J, Evans K, Goldman D, Martin G, Treco D, Heinemann S, Patrick J (1986) Isolation of a cDNA clone coding for a possible neural nicotinic acetylcholine receptor α-subunit. Nature 319:368–374.

Boulter J, Connolly J, 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 USA 84:7763–7767.

Brown DA, Fumagalli L (1977) Dissociation of α-bungarotoxin binding and receptor block in the rat superior cervical ganglion. Brain Res 129:165–168.

Buller AL, White MM (1990) Functional acetylcholine receptors expressed in *Xenopus* oocytes after injection of *Torpedo* β, γ and δ subunit mRNAs are a consequence of endogenous oocyte gene expression. Mol Pharmacol 37:423–428.

Carbonetto ST, Fambrough DM, Muller KJ (1978) Nonequivalence of α-bungarotoxin receptors and acetylcholine receptors in chick sympathetic neurons. Proc Natl Acad Sci USA 72:1016–1028.

Changeux J-P, Giraudat J, Dennis M (1987) The nicotinic acetylcholine receptor: molecular architecture of a ligand-regulated ion channel. Trends Pharmacol Sci 8:459–465.

Coleman A (1984) Translation of eukaryotic messenger RNA in *Xenopus* oocytes. In: Transcription and translation; a practical approach (Hames BD, Higgins J, eds), pp 271–302. Arlington, VA: IRL.

Colquhoun D, Ogden DC, Mathie A (1987) Nicotinic acetylcholine receptors of nerve and muscle: functional aspects. Trends Pharmacol Sci 8:465–472.

Deneris ES, Connolly J, Boulter J, Wada E, Wada K, Swanson LW, Patrick J, Heinemann S (1988) Primary structure and expression of beta2: a novel subunit of neuronal nicotinic acetylcholine receptors. Neuron 1:45–54.

Dennis M, Giraudat J, Kotzyba-Hibert F, Goeldner M, Hirth C, Chang J-Y, Lazure C, Chretien M, Changeux J-P (1988) Amino acids of the *Torpedo marmorata* acetylcholine receptor α subunit labelled by photoaffinity ligand for acetylcholine binding site. Biochemistry 27: 2346–2357.

Duvoisin RM, Deneris ES, Boulter J, Patrick J, Heinemann S (1989) The functional diversity of the neuronal nicotinic acetylcholine receptors is increased by a novel subunit: $\beta 4$. Neuron 3:487–496.

Goldman D, Deneris E, Luyten W, Kochhar 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.

Hartman DS, Claudio T (1990) Coexpression of two distinct muscle acetylcholine receptor α-subunits during development. Nature 343: 372–375.

Kao PN, Dwork AJ, Kaldany R-RJ, Silver MS, Wideman J, Stein S, Karlin A (1984) Identification of the α subunit half-cystine specifically labelled by an affinity reagent for the acetylcholine receptor binding site. J Biol Chem 259:11662–11665.

Koren G, Liman ER, Logothetis DE, Nadal-Ginard B, Hess P (1990) Gating mechanism of a cloned potassium channel expressed in frog oocytes and mammalian cells. Neuron 2:39–51.

Luetje CW, Patrick J (1989) Members of the neuronal nicotinic acetylcholine receptor family display distinct pharmacological and toxicological properties. Soc Neurosci Abstr 15:677.

Luetje CW, Wada K, Rogers S, Abramson SN, Tsuji K, Heinemann S, Patrick J (1990a) Neurotoxins distinguish between different neuronal nicotinic acetylcholine receptors. J Neurochem 55:632–640.

Luetje CW, Patrick J, Séguéla P (1990b) Nicotine receptors in the mammalian brain. FASEB J 4:2753–2760.

Mishina M, Takai T, Imoto K, Noda M, Takahashi T, Numa S, Methfessel C, Sakmann B (1986) Molecular distinction between fetal and adult forms of muscle acetylcholine receptor. Nature 321:406–411.

Moss BL, Schuetze SM, Role LW (1989) Functional properties and developmental regulation of nicotinic acetylcholine receptors on embryonic chicken sympathetic neurons. Neuron 3:597–607.

Neubig RR, Cohen JB (1979) Equilibrium binding of [^{3}H]tubocurarine and [^{3}H]acetylcholine by *Torpedo* postsynaptic membranes: stoichiometry and ligand interactions. Biochemistry 18:5464–5475.

Ogden DC, Colquhoun D (1985) Ion channel block by acetylcholine, carbachol and suberyldicholine at the frog neuromuscular junction. Proc R Soc Lond [Biol] 225:329–355.

Oortgiesen M, Vijverberg HPM (1989) Properties of neuronal type acetylcholine receptors in voltage clamped mouse neuroblastoma cells. Neuroscience 31:169–179.

Papke RL, Boulter J, Patrick J, Heinemann S (1989a) Single-channel currents of rat neuronal nicotinic acetylcholine receptors expressed in *Xenopus* oocytes. Neuron 3:589–596.

Papke RL, Duvoisin R, Boulter J, Heinemann S (1989b) The possible importance of the neuronal nicotinic subunit β4 to the kinetic properties of the adrenal chromaffin cell AChR. Soc Neurosci Abstr 15: 828.

Patrick J, Stallcup W (1977) α-Bungarotoxin binding and cholinergic receptor function on a rat sympathetic nerve line. J Biol Chem 252: 8629–8633.

Pedersen SE, Cohen JB (1990) *d*-Tubocurarine binding sites are located at the α-γ and α-δ subunit interfaces of the nicotinic acetylcholine receptor. Proc Natl Acad Sci USA 87:2785–2789.

Pritchett DB, Sontheimer H, Shivers BD, Ymer S, Kettenmann H, Schofield PR, Seeberg PH (1989) Importance of a novel $GABA_A$ receptor subunit for benzodiazepine pharmacology. Nature 338:582–585.

Schuetze SM, Role LW (1987) Developmental regulation of nicotinic acetylcholine receptors. Annu Rev Neurosci 10:403–457.

Sine SM, Steinbach JH (1984) Agonists block currents through acetylcholine receptor channels. Biophys J 46:277–284.

Sine SM, Steinbach JH (1986) Activation of acetylcholine receptors on clonal mammalian BC3H-1 cells by low concentrations of agonist. J Physiol (Lond) 373:129–162.

Sine SM, Steinbach JH (1987) Activation of acetylcholine receptors on clonal mammalian BC3H-1 cells by high concentrations of agonist. J Physiol (Lond) 385:325–359.

Sine SM, Taylor P (1980) The relationship between agonist occupation and the permeability response of the cholinergic receptor revealed by bound cobra α-toxin. J Biol Chem 255:10144–10156.

Sine SM, Taylor P (1981) Relationship between reversible antagonist occupancy and the functional capacity of the acetylcholine receptor. J Biol Chem 256:6692–6699.

Steinbach JH, Ifune C (1989) How many kinds of nicotinic acetylcholine receptor are there? Trends Neurosci 12:3–6.

Tate SS, Urade R, Micanovic R, Gerber L, Udenfriend S (1990) Secreted alkaline phosphatase: an internal standard for expression of injected mRNAs in the *Xenopus* oocyte. FASEB J 4:227–231.

Wada K, Ballivet M, Boulter J, Connolly J, Wada E, Deneris ES, Swanson LW, Heinemann S, Patrick J (1988) Functional expression of a new pharmacological subtype of brain nicotinic acetylcholine receptor. Science 240:330–334.