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ARTICLE *in* DEVELOPMENTAL GENETICS · JANUARY 1993

DOI: 10.1002/dvg.1020140407 · Source: PubMed

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DEVELOPMENTAL GENETICS 14:296–304 (1993)

Uptake of Antisense Oligonucleotides and Functional Block of Acetylcholine Receptor Subunit Gene Expression in Primary Embryonic Neurons

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ABSTRACT Several recent studies have used antisense oligonucleotides in the nervous system to probe the functional role of particular gene products. Since antisense oligonucleotide-mediated block of gene expression typically involves uptake of the oligonucleotides, we have characterized the mechanism of this uptake into developing neurons from embryonic chickens. Antisense oligonucleotides (15 mers) added to the bathing media are taken up into the embryonic chicken sympathetic neurons maintained in vitro. A portion of the oligonucleotide uptake is temperature dependent and saturates at extracellular oligonucleotide concentrations $\geq$ 20 μM. This temperature sensitive, saturable component is effectively competed by single nucleotides of ATP and AMP and is reminiscent of receptor-mediated endocytosis of oligonucleotides described in non-neuronal cells. The efficiency of the oligonucleotide uptake system is dependent on the developmental stage of the animal but independent of the number of days that the neurons are maintained in vitro.

Following the uptake of antisense oligonucleotides directed against ion channel subunit genes expressed by these neurons (nicotinic acetylcholine receptor subunit α3; nAChR α3), biophysical assays reveal that the functional expression of the target gene is largely blocked. Thus the number of wild type nAChR channels expressed is decreased by $\approx$80%–90%. Furthermore, following antisense deletion of α3, "mutant" nAChRs with distinct functional characteristics are expressed.

In sum, these studies characterize the uptake of antisense oligonucleotide and demonstrate the functional block of specific gene expression in primary developing neurons. In addition, the functional studies emphasize the need for sensitive and specific assay following antisense deletion, since other homologous gene products may substitute for the targeted gene resulting in new phenotypes that are subtly different from wild type.

Key words: Transport, nicotinic acetylcholine receptor, sympathetic neurons

INTRODUCTION

Antisense oligonucleotides have become useful tools for studies of gene function. The use of antisense oligonucleotides as possible therapeutic agents is currently being explored in detail, since they can selectively modify the expression of a single targeted gene. It is hypothesized that antisense oligonucleotides can hybridize with target sequences on mRNA thereby preventing the translation of the messenger RNA [Aktar and Juliano, 1992]. Alternatively, oligonucleotides might form a triple helix complex with specific regions of double stranded DNA, thereby blocking target gene transcription [Aktar and Juliano, 1992]. Characterization of the uptake of oligonucleotides into several cell lines has revealed that despite their size and hydrophilic nature, oligonucleotides of 25 bases or less can enter cells, albeit slowly. By using acridine as a fluorescent marker at the 5′ end of a D-oligo homopolymer, Loke and collaborators have shown that the uptake of oligonucleotides into HL60 cell line is saturable and temperature dependent. Subsequent work demonstrated that unmodified DNA oligonucleotides (D-oligo) as well as phosphorothioate oligonucleotides(S-oligo) are taken up into various cells lines via receptor mediated endocytosis [Loke *et al.*, 1989; Stein *et al.*, 1988]. A distinct mechanism, however, may mediate the uptake of other modified oligonucleotides such as

Received for publication March 12, 1993; accepted April 26, 1993.

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the methylphosphonate (MP) oligonucleotides [Akhtar *et al.*, 1991b]. An 80 kD protein has been identified as a putative receptor for D-oligo uptake by oligo(dT)-cellulose affinity chromatography. The existence of a cell surface receptor for D-oligo was confirmed by an independent approach where the 5′-end of D-oligo was conjugated with a reactive alkylating group to crosslink the oligonucleotides to mouse fibroblast L929 cells [Yakubov *et al.*, 1989]. Two putative receptor proteins (79 kD and 90 kD) were labeled and indicated the expression of ≈120,000 receptors per L929 cell.

In contrast to these studies in muscle and fibroblast cell lines, however, relatively little is known about the process of oligonucleotide uptake into primary cells and neurons. Furthermore, the possibility of changes in oligonucleotide uptake with neuronal development have not been explored. It is clearly important to define reliable conditions for administration and targeting of specific oligonucleotides in the developing nervous system. To this end, we examined the oligonucleotide uptake process in embryonic neurons.

In earlier studies, we explored the use of antisense oligonucleotides to attempt a dissection of a particular transmitter-gated ion channel [Listerud *et al.*, 1991]. This approach was taken to examine the functional role of individual subunits of a receptor channel complex within its native environment. All ligand-gated ion channels studied to date are multimeric protein complexes composed of several subunits encoded by homologous genes. In most of the ligand-gated ion channel families thus far identified (including GABA receptors, NMDA receptors, AMPA/KA receptors, glycine receptors, and neuronal nicotinic ACh receptors) there are several homologous versions of each subunit type that could contribute to the formation of a diverse array of channels in vivo [for review see Unwin, 1993]. In the case of neuronal nicotinic ACh receptors, the channel complex includes 5 subunits, composed of two subunit types (α and β) that are encoded by at least 11 related genes [see Role, 1992; Sargent, 1993, for recent reviews]. In embryonic chick sympathetic ganglion neurons, four channel types and the expression of six subunit genes are detected (α3, α4, α5, α7, β2, and β4 [Moss *et al.*, 1989; Listerud *et al.*, 1991; Moss and Role, 1993; Devay *et al.*, 1993]). Expression of particular combinations of the subunit genes in *Xenopus* oocytes yield biophysically and pharmacologically distinct channels. However, the heterologously expressed nAChRs reported to date do not match those expressed in vivo. Since these individual subunit genes are differentially expressed in distinct neuron populations and the patterns of gene expression are regulated during development, it is clearly essential to probe the functional role of individual subunits in their native environment—that is, in primary neurons. Antisense deletion of individual subunits has begun to yield information relevant to this "in vivo" channel dissection in developing neurons [Listerud *et al.*, 1991].

Although several groups have used antisense oligonucleotide techniques to examine the functional contribution of various proteins within neuronal cell lines [Caseres and Kosik, 1990], there is little known about the uptake system in primary neuronal cells [Listerud *et al.*, 1991; Lallier and Bronner-Fraser, 1993; Wahlestedt *et al.*, 1993]. To better understand the mechanisms of antisense oligonucleotide block of gene expression, we characterized the process of oligonucleotide uptake into primary neurons at different stages of neuronal development. We found robust temperature dependent and saturable uptake of short oligonucleotides (15 mers) into neurons throughout embryogenesis. Furthermore, we confirm that these oligonucleotides, if appropriately directed to particular subunit gene target sequences, can lead to specific functional block of subunit expression.

MATERIALS AND METHODS

Tissue Culture

Cultured chick sympathetic neurons were obtained by dissecting the lumbar sympathetic chain ganglia from chicks of different embryonic ages (ED11–17), dispersing the ganglia into individual cells and plating under conditions that suppress the proliferation of nonneuronal cells. The culture medium was Eagle's Minimal Essential Medium (MEM) supplemented with 10% heat-inactivated horse serum/5% chicken embryo extract/penicillin (500 μg/ml)/streptomycin (50 μg/ml)/2.4 mM glutamine/2.5S nerve growth factor (0.5 μg/ml). Details of the cell culture protocol have published previously [Role, 1984; with modifications as outlined in Gardette *et al.*, 1991]. As an additional measure to prevent the growth of nonneuronal cells in cultures of older embryonic tissue, the initial cell suspension was γ-irradiated (≈5,000 rads) just prior to plating. Cells were plated at a density of 0.5 chains in 0.5 ml media in 24 well polyornithine coated Falcon tissue culture plates.

Quantification of Oligonucleotide Uptake

In most experiments, the oligonucleotide used for the uptake studies was a 15-mer D-oligonucleotide designed as antisense to the region of the α3 subunit start site. The sequence of this oligo is 5′-GGACCATAAACCAAC-3′, synthesized by Genosis, Inc. In a few preliminary experiments the sense orientation oligonucleotide to this sequence was tested for uptake into cells with identical results. Oligonucleotides are 5′ end labeled with ^{32}P-γ-ATP by T4 polynucleotide kinase (Promega) in kinase buffer (Promega) and subsequently purified by column chromatography on C_{18} "Sep-Pak" columns (Waters). Oligonucleotides were used within 24 hours of end-labeling. For each experiment radioactive oligonucleotides are mixed with unlabeled oligonucleotide in a ratio of 1:20 so that the specific activity remained constant throughout. The ra-

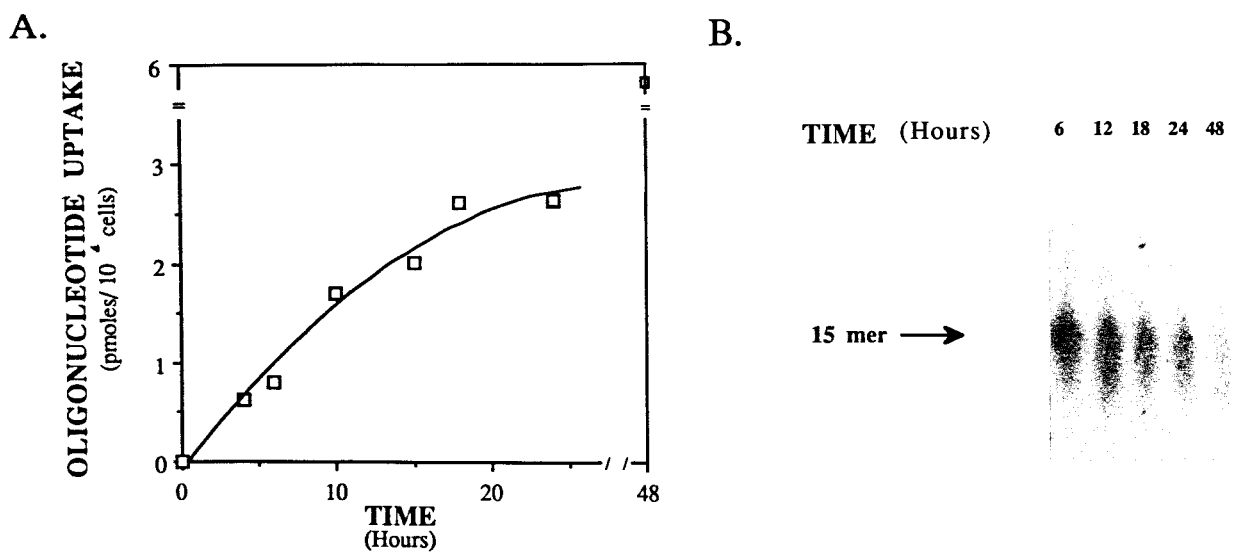


Fig. 1. Time course of oligonucleotide uptake. ^{32}P-γ-ATP labeled 15 mer D-oligonucleotides were added directly to the bathing media together with unlabeled oligonucleotides at a final concentration of 10 μM. Cells were washed and lysed at different times after incubation at 37°C. Total CPM were counted and the amount of oligonucleotide associated with the cells was calculated. ^{32}P labeled oligonucleotide bound to the dish and cell membranes were determined and subtracted as described in Materials and Methods. **A:** Total oligonucleotide uptake into the cells at the indicated times. **B:** Autoradiogram of ^{32}P-labeled material within the cells at the indicated times. Note that intact 15 mer is still detected at 48 hours.

dioactive/non-radioactive oligonucleotide mixture is then dissolved in experimental media to the desired concentration (0–50 μM) and incubated with primary sympathetic neurons in vitro for the time specified. After incubation, neurons are washed free of extracellular oligonucleotide by 3 washes with non-radioactive washing buffer (144 mM NaCl, 5 mM Tris, 2% BSA, pH 7.4) and then lysed with 0.5 mM Tris (pH 7.4), 0.1 mM EDTA, 25% NP40. The cell lysates are collected and radioactivity quantified by scintillation spectrometry (Beckman) in 3 mls of scintillation fluid. The total oligonucleotide associated with the cells is calculated from the specific activity of the added material. An aliquot of the cell lysate is extracted with phenol and chloroform and dried overnight in an Eppendorf Speed-Vac (Model SC 110). The dried samples are resuspended in TE and loaded on denaturing polyacrylamide gel to determine the oligonucleotide size. In some experiments the quantity of material migrating as 15 mer is quantified by excision and assay of the band by liquid scintillation spectrometry.

To evaluate the contribution of non-specific binding of oligonucleotide, sibling culture are incubated with labeled oligonucleotide for 5 minutes at 4°C followed by repeated buffer wash at 4°C and then extracted. The counts were quantified and subtracted as background from the samples throughout, unless otherwise indicated.

Electrophysiological Recording

Tight seal whole cell recording and single channel recording employed the patch clamp technique [Hamill *et al.*, 1981]. Patch clamp electrodes were obtained by a two-stage pull on a vertical electrode puller (Kopf 700D or Narashige PP-83) and electrodes were filled with 150 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES, 5 mM Mg-ATP, and 1 mM EGTA at pH 7.2 for whole cell recording and outside-out single channel recording, or with 140 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM HEPES at pH 7.2 with acetylcholine (ACh; 1-25 μM) for cell attached single channel recording. In all functional assays both control and experimental cells were pretreated with bromo-acetylcholine-bromide (BAC) as previously described [Gardette *et al.*, 1991]. The irreversible block of surface nAChRs by BAC eliminates the contribution of nAChRs expressed prior to antisense treatment to subsequent biophysical recordings. For physiological recording experiments neurons are plated in 35 mm dishes with a 13-mm diameter sunken well of ≈150 μl volume to conserve the amount of oligonucleotide used.

RESULTS

Time Course of Oligonucleotide Uptake

The time course of uptake of a labeled 15-mer oligonucleotide (an antisense oligonucleotide to the nAChR subunit gene α3; see Materials and Methods) into ED11 chick sympathetic neurons in vitro is illustrated in Figure 1A. In these experiments, the oligonucleotide previously labeled with ^{32}P as described in Materials and Methods, is added at a final concentration of 10 μM to neurons that had been maintained in vitro for 3–4 days. This is the concentration of oligonucleotide typi-

cally used in functional assays of antisense oligonucleotide block [see below and Listerud *et al.*, 1991]. Following the incubation with labeled oligonucleotide, the neurons are washed and lysed at the indicated times and the total amount of uptake determined. The total amount of oligonucleotide associated with the cells continuously increased early on (1–12 hours) and then gradually approached a plateau at ≈20 hours. Examination of neuronal extracts by PAGE revealed that the amount of intact 15 mers in the cells is maximal by 6 hours, declining thereafter. At 48 hours of incubation with oligonucleotide, total oligonucleotide uptake is maximal and intact, free, 15 mer can still be detected in the cells (Fig. 1B).

The time course experiments also revealed the rate of oligonucleotide degradation in the culture medium. Oligonucleotides of <15 bases in length are detected by PAGE within 3 hours of incubation and after 48 hours, the amount of intact 15-mer in the medium is barely detectable. The oligonucleotide degradation is reduced by two fold by prior heat inactivation of added components including the chick embryo extract (CEE; preincubation at 68°C for 1.5 hours; data not shown).

Concentration Dependence of Oligonucleotide Uptake

The concentration dependence of oligonucleotide uptake into embryonic chick sympathetic neurons (ED11) was determined after 6 hours of incubation with labeled oligonucleotide from 0 to 50 μM (Fig. 2). At 37°C there is nearly a linear increase of total oligonucleotide associated with the cells with increasing concentration of oligonucleotide. However, a linear increase is also observed if cells are incubated at 4°C, indicating that there is a significant portion of the oligonucleotide associated with the cells in a temperature independent manner. The difference between the uptake under these two conditions reveals the extent of the temperature dependent component of oligonucleotide uptake. This component saturates at ≈20 μM. PAGE autoradiograms of the cell extracts reveal a strong band of 15 mer at this concentration, indicating that much of the oligonucleotide is intact. Assuming that the cells are reasonably spherical with a diameter of about 30 μm, the intracellular concentration of oligonucleotide has nearly reached equilibrium with the external concentration of oligonucleotide by six hours of incubation with 10 μM oligonucleotide. A decrease in the external concentration of oligonucleotide to 5 μM decreases the calculated internal concentration by 70% (from 9 μM to 3 μM). Increasing external oligonucleotide concentration to >20 μM has little effect on increasing internal concentration.

Competition of Oligonucleotide Uptake by Single Nucleotides

To further investigate the mechanism of oligonucleotide uptake into neurons and to compare with previous studies of oligonucleotide uptake into non-neuronal cell lines, we examined the competition of oligonucleotide uptake by single nucleotides. Figure 3 summarizes data indicating that both ATP and AMP can compete for oligonucleotide uptake. This result, in combination with those above, suggests that oligonucleotide uptake is mediated by a specific, temperature dependent and saturable process. Our finding that single nucleotides can compete with oligonucleotide uptake is consistent with previous studies of the oligonucleotide receptors (transporters) on non-neuronal cell lines [Loke *et al.*, 1989; Yakubov *et al.*, 1989]. Thus it seems likely that the temperature sensitive, saturable

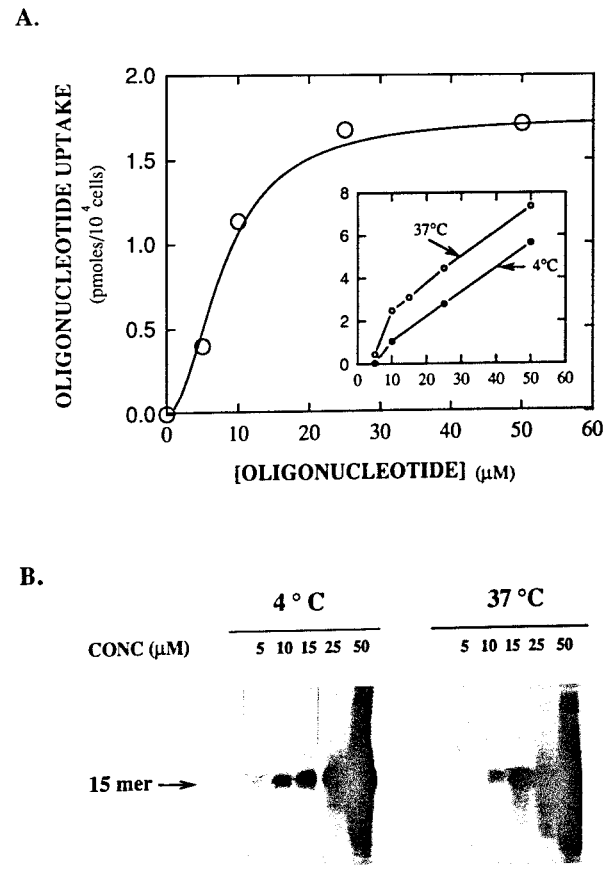


Fig. 2. Concentration dependence of oligonucleotide uptake. After 6 hours of incubation with the indicated concentrations of oligonucleotides at either 4°C or 37°C, samples were assayed and calculated for the total amount of oligonucleotide uptake as described in Materials and Methods. **A:** The temperature dependent portion (37°C–4°C) of oligonucleotide uptake is plotted vs. the external oligonucleotide concentration. Uptake saturates at ≈20 μM. Inset: data at both 37°C and 4°C with subtraction of non-specific binding. Both showed a nearly linear increase with increasing extracellular oligonucleotide concentration. **B:** Autoradiograph of oligonucleotide associated with the cells at both temperatures.

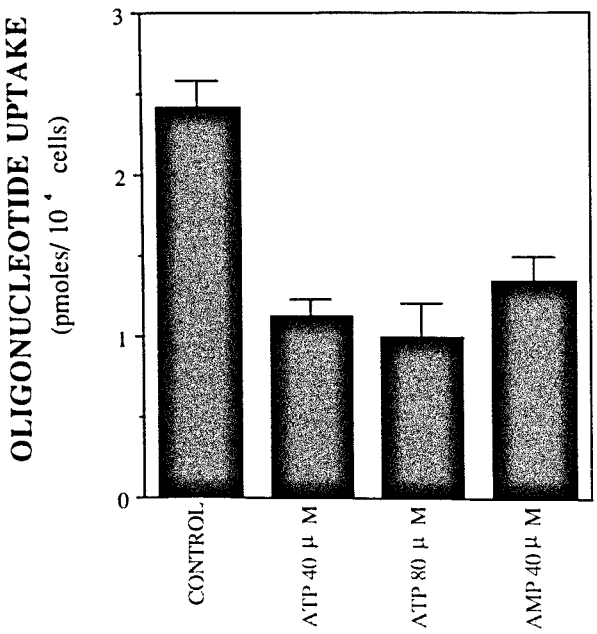


Fig. 3. Competition of oligonucleotide uptake by nucleotides. Cells were incubated with 10 μM oligonucleotide and either ATP or AMP at the specified concentrations for 6 hours at 37°C. ATP and AMP apparently compete with oligonucleotides for uptake, detected as a decrease in oligonucleotide taken up by the cells (data subtracted for nonspecific binding: see Materials and Methods).

component of oligonucleotide uptake is a receptor-mediated process.

Changes in Concentration Dependence and Extent of Oligonucleotide Uptake During Embryogenesis

The developmental changes in the concentration dependence of oligonucleotide uptake was examined by measuring uptake into both ED11 and ED17 sympathetic neurons (Fig. 4A). At low concentrations (5 and 10 μM), the ED17 neurons show about twofold more uptake per cell than ED 11 neurons. At high concentrations, however, there is no significant difference with embryonic age of the neurons. Thus, although the uptake at submaximal concentration is slightly enhanced during development, there is apparently no change in the maximal uptake (>25 μM). The neurons increase in both volume and surface area during development by about 1.5-fold (as calculated from cell capacitance determinations; Moss and Role, 1993). Thus the density of oligonucleotide uptake sites, as indicated by uptake at maximal oligonucleotide concentrations, apparently decreases at later developmental stages (Fig. 4B). Assay of ED11 neurons maintained in vitro for 5–6 days were comparable in uptake to those maintained for 3–4 days, suggesting that these changes in uptake are not acquired with development in vitro (not shown).

The Uptake of Specific Antisense Oligonucleotides Leads to Functional Block of the Targeted Product

We have used a physiological rather than biochemical assay to determine whether a specific oligonucleotide, once taken up into these embryonic neurons, can block the functional expression of the targeted gene. In these experiments we used an antisense oligonucleotide directed against one of the subunits of the AChR channels expressed in these cells (an antisense to the α3 subunit, the same used for most uptake studies; see Materials and Methods). We then evaluated the specificity of antisense oligonucleotide-mediated block by patch clamp assay of AChR channel currents. After four days in vitro, neurons were treated with bromoacetylcholine (BAC) to block the pre-existing surface AChR channels, and then incubated with 10 μM α3 antisense oligonucleotide for 24–48 hours. We then used both single channel recording and tight seal whole cell recording to evaluate the extent of functional block by α3 antisense oligonucleotide following their uptake (Fig. 5).

A typical record of single channel activity gated by 2.5 μM ACh in a control neuron is shown in Figure 5A. Dishes of control neurons were handled identically to experimental dishes, except for incubation with either an α3 sense oligonucleotide or a three base mismatch antisense sequence [see Materials and Methods and Listerud *et al.*, 1991]. Comparable recordings from neurons previously treated with α3 antisense reveals a 80–90% decrease in the number of channel openings gated by agonist (Fig. 5A′). The observed decrease is apparently due to both a shift in the ACh concentration required to gate channel activity and a decrease in the total number of functional channels expressed following α3 antisense treatment. Thus, when a higher concentration of agonist (25 μM ACh) is used, it is apparent that the α3 antisense oligonucleotide treated neurons are expressing ACh-activatable channels.

Single channel recordings also revealed that the nAChRs in α3-deleted neurons desensitize more slowly than control nAChRs activated by high agonist concentration (Fig. 5B,B′). Application of 25 μM ACh to outside-out patches from control neurons evoked a burst of channel openings followed by a rapid decrease in opening frequency so that within <10 seconds few openings are seen (Fig. 5B). In contrast, the same concentration of ACh applied to a patch excised from α3-deleted neurons evoked persistent channel openings so that robust activity is still observed at 10–20 seconds of agonist exposure (Fig. 5B′). This slowing of AChR desensitization is also seen at the macroscopic current level (Control: τ_f ranged from ~0.4 to 1.4 seconds, τ_s ranged from 5 to 8 seconds, n = 27, vs. α3 antisense treated: τ_f 1.4–2.9 seconds τ_s 11–19 seconds, n = 26; Fig. 5C,C′ [and Listerud et al., 1991].

The decrease in the total number of functional

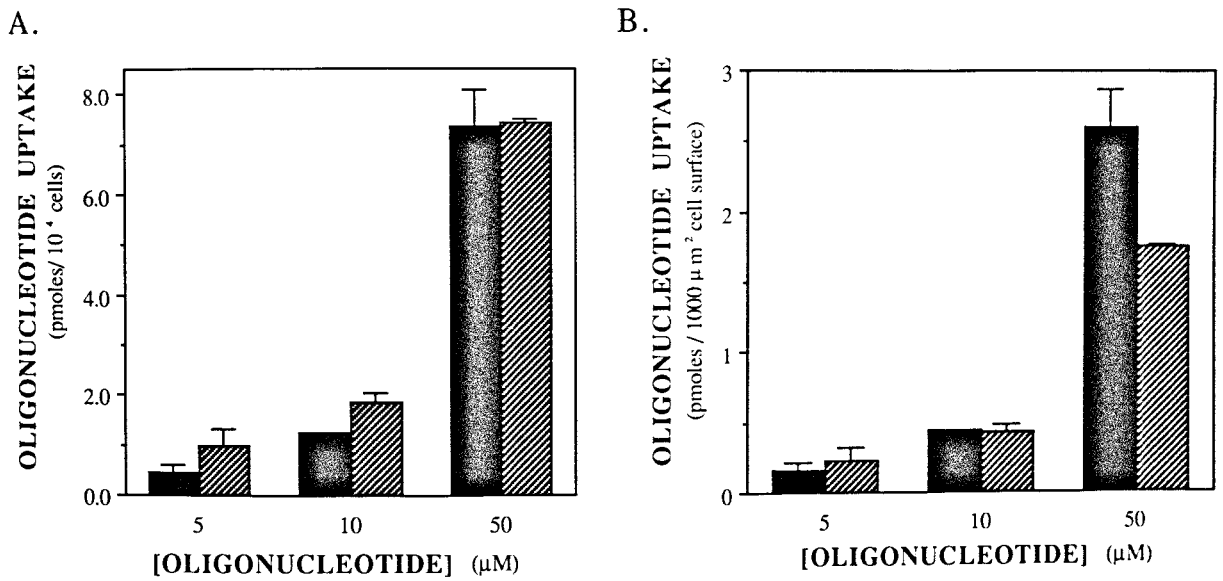


Fig. 4. Developmental changes in oligonucleotide uptake. Concentration dependence of oligonucleotide uptake into neurons removed at either ED 11 (solid bars) and ED 17 (hatched bars) and maintained in vitro for 3–7 days, as described in Materials and Methods. **A:** Oligonucleotide uptake per cell is significantly greater in ED 17 neurons compared with ED 11 neurons when 5 or 10 μM external oligonucleotide is used ($P < 0.1$), whereas uptake assayed at 50 μM shows no difference. This indicates that maximal uptake is comparable on a per neuron basis at ED 11 and ED 17. **B:** Correction of uptake for cell surface area reveals that the density of oligonucleotide uptake sites is about two fold greater in younger neurons. In view of the increase in cell volume with age, the net concentration of oligonucleotide at subsaturating concentrations is comparable, but at saturating concentrations is ≈2 fold greater in ED 11 neurons.

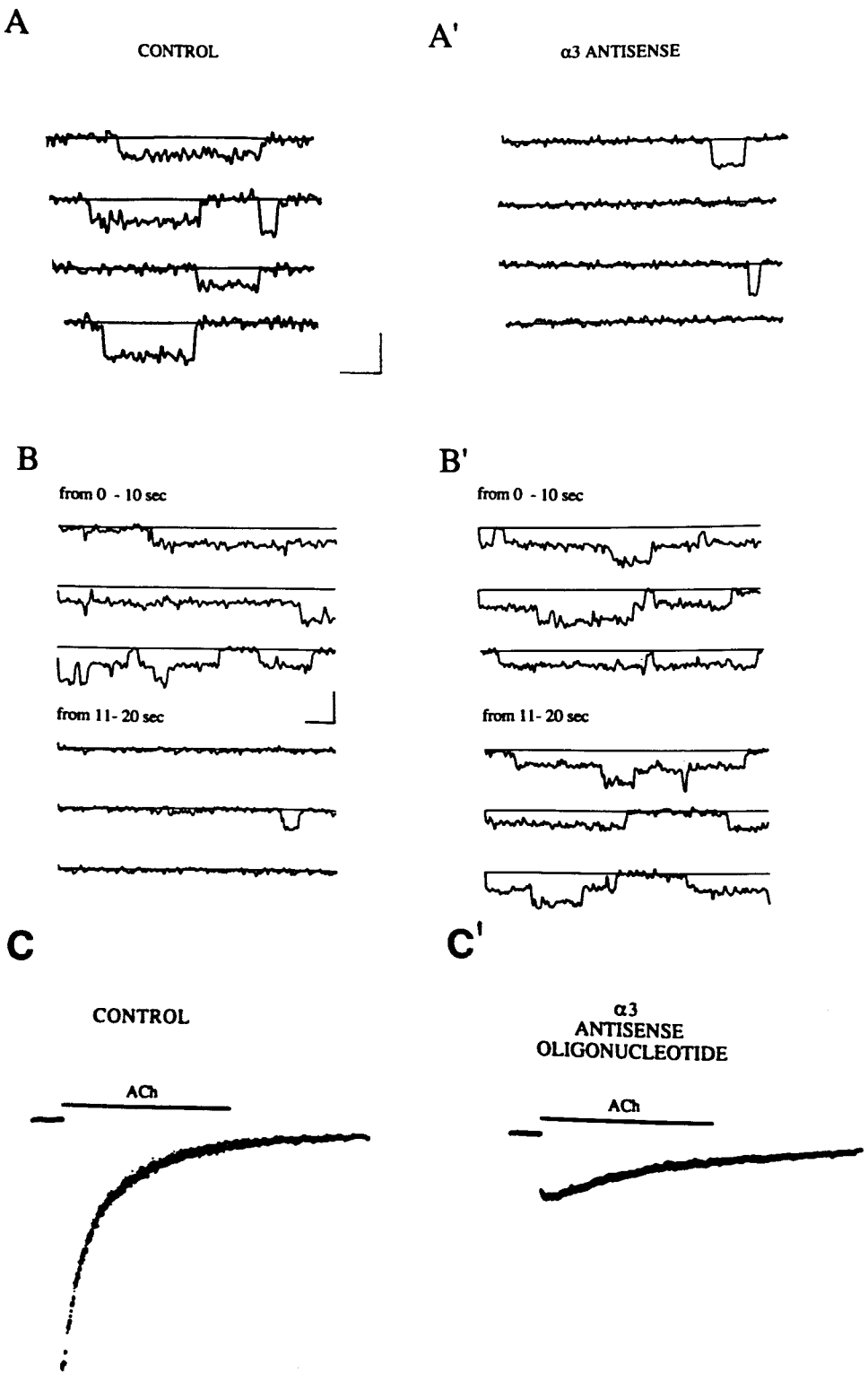
AChRs is also revealed by assay of the response to maximal concentrations of ACh at the macroscopic current level. Following α3 antisense treatment, the peak response to maximal ACh (250 μM) is significantly reduced (Fig. 5C′). Recent experiments indicate that the slowly desensitizing ACh-evoked currents are novel nAChR subtypes comprised, at least in part, of distinct but homologous nAChR subunits [Listerud *et al.*, 1991; Yu and Role, in preparation].

DISCUSSION

Despite the increasing use of antisense oligonucleotides to examine gene function, the regulation of oligonucleotide transport into primary embryonic neurons has not been studied in detail [Listerud *et al.*, 1991; Lallier and Bronner-Fraser, 1993; Wahlestedt, 1993]. The goals of this study were to characterize the mechanism of antisense oligonucleotide uptake into primary neuronal cells and document that antisense oligonucleotide taken up into neurons are capable of interfering with gene expression. We find that peripheral neurons take up oligonucleotide of 15 bases in length in a temperature dependent, saturable manner that is competitively inhibited by single nucleotides (e.g., ATP, AMP). Overall, the properties of oligonucleotide uptake into primary embryonic neurons are consistent with receptor-mediated endocytosis, the mechanism that is apparently utilized by non-neuronal cells to take up oligonucleotide of various length and composition [see Aktar and Juliano, 1992, for review].

To further confirm the efficacy of the oligonucleotide used in uptake studies, we treated cultured sympathetic neurons with an antisense oligonucleotide directed against the start region of a gene encoding a prominent nAChR subunit (α3). When the neurons are incubated at a concentration close to saturation α3-antisense oligonucleotides reduce the macroscopic response to maximal concentrations of ACh, indicating a decrease in the total number of functional AChR channels. Furthermore, the channels expressed have biophysical properties distinct from wild-type nAChR channels. Neither sense orientation or 3 base mismatched oligonucleotides affect nAChR channel properties (not shown). These data indicate that the uptake of oligonucleotides into developing neurons results in their targeting to the synthetic machinery such that the appropriate gene is functionally deleted.

It should be noted that with an assay less sensitive than single channel recording, the efficacy of the antisense treatment would have been underestimated. Thus analysis of the differences in macroscopic current suggest that only 50–60% of the total current is blocked. This estimate is distorted, however, by the significant contribution of nAChR channels that require high agonist concentrations for gating and that desensitize slowly, unlike the wild-type channels. Ex-


A
CONTROL
A'
α3 ANTISENSE
B
from 0 - 10 sec
from 11- 20 sec
B'
from 0 - 10 sec
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C
CONTROL
ACh
C'
α3
ANTISENSE
OLIGONUCLEOTIDE
ACh

Fig. 5.

amination at the single channel level reveals that 80–90% of the wild-type channels are deleted. Other studies have shown that α7, a subunit homologous to α3 but pharmacologically distinct, is a major ligand binding subunit in these novel channels [Listerud *et al.*, 1991; Yu and Role, in preparation].

Several characteristics of the oligonucleotide uptake into neurons are unusual. For example, close inspection of the concentration dependence of the temperature sensitive component reveals a steep increase in oligonucleotide uptake between 5 and 10 μM. The difference in net uptake with further increases in oligonucleotide concentration >10 μM is relatively small. Consistent with the steep concentration dependence of the uptake is the equally steep concentration dependence of functional block of gene expression. Thus use of 5 μM α3 antisense oligonucleotide in the functional assay had little effect on the peak of the whole cell current (data not shown). Cells treated with 10 μM vs. 20 μM antisense oligonucleotide were similarly affected, as quantified at the macroscopic level. Calculation of the relative concentration of oligonucleotide in the cells vs. in the media revealed a threefold difference between cells incubated with 10 μM vs. 5 μM oligonucleotide; apparently, a critical difference in oligonucleotide efficacy.

Antisense oligonucleotides presumably interfere with gene expression by hybridizing to a specific region of the mRNA or DNA encoding the target gene. The extent of degradation of oligonucleotides in both the medium and inside the cell may significantly alter the efficacy of antisense block by regulating the concentration of full length oligonucleotide available for hybridization and block. More oligonucleotide may not necessarily be better, since high concentrations of oligonucleotide will increase the concentration of smaller (non-specific) oligonucleotide fragments taken up that can result in non-specific effects and even cause cell death. In fact, these results underscore the critical concentration dependence of antisense oligonucleotides for effective and specific block of gene expression. Unfortunately, this critical concentration dependence may differ from cell type to cell type and is certainly regulated with development stage (Fig. 4). Our data indicate that for cultured chick sympathetic ganglion neurons at young embryonic ages, 10 μM oligonucleotide is both necessary and sufficient to functionally delete the receptor subunit genes.

To our surprise, the temperature dependence of the uptake is quite different from what is seen in non-neuronal cells. In our study, the difference in absolute amount of oligonucleotide associated with cells at 37°C vs. 4°C is relatively small. In contrast, studies in non-neuronal cells have indicated substantial differences in oligonucleotide uptake over this same temperature range. There are several possible explanations. The most obvious is that neuronal cells might bind oligonucleotide with higher affinity than non-neuronal cells, so that the background "uptake" (binding) will be considerably higher. In our study, we assume that binding of oligonucleotide to cell surface is complete within 5 minutes at 4°C, whereas uptake under these conditions is negligible. Assuming a larger number of sites with slow K_{on} and K_{off} for oligonucleotide binding, the oligonucleotide associated with neurons after 5 minutes at 4°C would be due to surface binding. Previous work by Aktar and collaborators, indicated that high affinity sites might arise, at least in part, from interaction of antisense oligonucleotides with phospholipid membrane [Akhtar *et al.*, 1991a]. Oligonucleotides enter liposomes very slowly ($t_{1/2}$ = 7 days), and the affinity of oligonucleotide for the lipid is very high [Kd ≈20 nM; see Aktar *et al.*, 1991a]. However, since we have used oligonucleotide concentrations ≈1,000 times the Kd of adsorption, it is unlikely that binding directly to the lipid would result in a linear increase of oligonucleotide with increasing oligonucleotide concentration at 4°C. Some protein component of high affinity and low on-off rate to oligonucleotide is likely to be involved and binding studies to neuronal membranes should resolve this issue. Examination of oligonucleotide uptake in the older neurons reveals a decrease in maximal uptake of oligonucleotide (Fig. 4B). The developmental regulation of the transport system may have some functional role; perhaps nucleotide uptake is utilized as a salvage mechanism early on. With the maturation and growth of the neurons, the density of uptake sites may decline, compensated, at least in part, by an increase in the efficiency of uptake at low concentrations. Further binding assay of oligonucleotides to the receptors expressed at the two ages of embryonic neurons could confirm this speculation.

Fig. 5. Antisense oligonucleotide can specifically affect functional properties of the targeted gene product. α3 antisense oligonucleotide (10 μM) was added to the culture media directly after preblock of surface nAChRs with BAC (see Materials and Methods). After 48 hours of incubation, electrophysiological experiments were conducted. **A, A′:** Single channel records ± α3 antisense pretreatment: low agonist concentration. Continuous records from single channel recordings of untreated and α3 antisense oligonucleotide treated cells with 2.5 μM ACh. The overall channel activity is reduced by about 90% in the antisense treated cells. **B, B′:** Single channel records ± α3 antisense pretreatment: high agonist concentration. Continuous records of single channel recording with 25 μM ACh from untreated and α3 antisense treated cells. 25 μM ACh gates significant channel activity in antisense treated neurons, but the channels gated differ from control in requirement for high agonist concentration and their slow desensitization. In control cells, nAChR channels desensitize very rapidly (1–2 seconds) after application of 25 μM ACh. **C, C′:** Macroscopic record ± α3 antisense pretreatment. Macroscopic currents evoked by maximal concentrations (250 μM) ACh in control neurons decay rapidly after reaching a peak of ≈500 pA (± 15%). Population study of neurons pretreated with antisense to α3 subunit reveals about 60% decrease in the peak current and ≈3-fold slower of desensitization time course.

ACKNOWLEDGMENTS

The authors thank Dr. R. Axel, Dr. D. Colman and Dr. J. Pintar for reagents, access to equipment, and helpful suggestions. This work was supported by grant award NS29071 to L.W.R.

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