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Differential Expression of Sodium Channels and Nicotinic Acetylcholine Receptor Channels in nnr Variants of the PC12 Pheochromocytoma Cell Line

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Abstract. An important component of neuronal differentiation is the tightly controlled expression of a spectrum of ion channel proteins. Ion channels play a critical role in the generation and propagation of action potentials as well as in the cellular response to neurotransmitters, and thus are central in the transfer and integration of information in the nervous system. A model system amenable to analysis of ion channel expression and neuronal differentiation is the rat pheochromocytoma (PC12) cell line. Here, we have used electrophysiological and molecular biological approaches to analyze the expression of voltage-dependent sodium (Na) channels and nicotinic acetylcholine receptors (nAChR) in mutagenized variants (nnr cells) of the PC12 cell line. Our data reveal striking differences in the expression of these channels when compared to wild-type PC12 cells. Even in the absence of nerve growth factor (NGF), nnr cells express functional Na channels and Na channel mRNA at levels exceeding those in wild-type PC12 cells differentiated with NGF. In contrast, acetylcholine-induced currents were evident in only a small proportion of cells, presumably due to the altered pattern of expression of mRNAs encoding individual nAChR subunits. The altered ion channel expression in these variants provides an avenue for analyzing Na channel and nAChR channel function, as well as for identifying mechanisms governing their expression.

Key words: Sodium channels — Acetylcholine receptor channels — Patch clamping — Gene expression — PC12 cells — Regulation

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Introduction

The transfer and integration of information in the nervous system depends upon the ability of individual neurons to generate and propagate electrical signals as well as to respond to neurotransmitters. As a result, a critical component of neuronal development and differentiation is the expression of a spectrum of ion channel proteins, including voltage-dependent sodium (Na) channels and nicotinic acetylcholine receptors (nAChR). The principal component of voltage-dependent Na channels is an ~260 kDa α subunit, and in the nervous system there are at least four α subunit genes (type I, II, IIa, III) that have been shown to encode functional Na channels (for reviews, *see* Catterall, 1992; Mandel, 1992). For nAChR, a large family of α and β subunit genes encode individual subunits which are assembled into functional receptors thought to consist of two α and three β subunits (for review, *see* Deneris et al., 1991). Although expression of ion channels appears to be very tightly regulated during development (O'Dowd, Ribera & Spitzer, 1988; Corriveau & Berg, 1993; for reviews *see* Spitzer 1985; Ribera & Spitzer, 1992; Sargent, 1993), the biochemical mechanisms that govern the expression of neuronal ion channels and determine the combinaton of voltage-dependent and ligand-activated channels a particular neuron will express are not well understood.

An extremely useful model for investigating neuronal differentiation and the development of electrical membrane properties is the rat pheochromocytoma (PC12) cell line (Greene & Tischler, 1976), which expresses the p75 and trk receptors for nerve growth factor (NGF) (Radeke et al., 1987; Kaplan et al., 1991) and in response to NGF adopts a sympathetic neuronlike phenotype (for reviews, *see* Greene & Tischler, 1982;

Guroff, 1985). PC12 cells have been used to study the functional properties, growth factor induction, and in some cases even the localization, of voltage-dependent Na channels, calcium channels, potassium channels, and nAChR channels (Dichter, Tischler & Greene, 1977; Streit & Lux, 1987; Garber, Hoshi & Aldrich, 1989; Ifune & Steinbach, 1990; Ginty et al., 1992; Fanger et al., 1993; Sharma et al., 1993; Henderson et al., 1994; Hu et al., 1994; for review, *see* Shafer & Atchison, 1991). In addition to the electrophysiological, biochemical, and molecular biological approaches utilized in these studies, PC12 cells are also amenable to genetic approaches, which have been used to isolate mutant sublines (Bothwell, Schechter & Vaughn, 1980; Burstein & Greene, 1982; Green et al., 1986), including NGF nonresponsive (nnr) variants (Green et al., 1986). These variants, which express p75, but no detectable level of trk (Loeb et al., 1991), have been widely used in trying to understand the cellular and molecular mechanisms underlying neuronal differentiation and the actions of NGF (Altin et al., 1991; Loeb et al., 1991, 1994; Loeb & Greene, 1993; Stephens et al., 1994).

We have analyzed ion channel expression in a variety of nnr subclones as part of an effort to identify mechanisms governing neuronal ion channel expression, especially with regard to the actions of NGF. Surprisingly, we find the expression of Na channels and nAChR channels in the nnr cells is substantially altered in comparison to what we have typically observed in wild-type PC12 cells. The results obtained in these variants emphasize that distinct mechanisms regulate the expression of Na channels and nAChR, and identify clonal cell lines that may serve as useful reagents for understanding cellular and molecular mechanisms governing the expression and function of these two classes of ion channel proteins.

Materials and Methods

Cell Culture

Cells were maintained in a humidified CO_2 environment in DME containing 0.45% glucose, 10% fetal bovine serum, 5% heat inactivated horse serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO, Grand Island, NY). Media for the stably transfected nnr subclones (T1, T9, T14, T8) also contained 500 μg/ml G418 (GIBCO). Media were changed every other day and cells were passaged once a week. Cells were grown in 100 mm (for RNA and transfection) or 35 mm (for electrophysiology) tissue culture dishes (Falcon Labware, Becton Dickinson, Lincoln Park, NJ). When appropriate, cells were treated with 100 ng/ml 7S NGF (Upstate Biotechnology, Plattsburg, NY) for 7 days, with fresh NGF added each time the media was changed.

Electrophysiological Recording and Analysis

Whole-cell Na currents and ACh-induced currents were assayed as in our earlier studies (Ginty et al., 1992; Fanger et al., 1993; Henderson et al., 1994). During recording, a 0.5 ml chamber containing the cells was continuously perfused at 1 ml/min with an extracellular saline solution ((mM) 150 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, 5 glucose). Patch-clamp recordings (Hamill et al., 1981) were made at room temperature (20–24°C) using a List EPC-7 patch-clamp amplifier (Medical Systems, Greenvale, NY). Patch electrodes were pulled from capillary glass (Sutter Instruments, Novato, CA) and had resistances of 3–7 MΩ when filled with an internal saline solution designed to minimize the contribution of other voltage-activated currents ((mM) 140 CsCl, 10 EGTA, 10 HEPES, pH 7.2). Puffer electrodes with tip diameters of 5–10 μm contained ACh (acetylcholine chloride; Sigma Chemical, St. Louis, MO) dissolved in the extracellular saline solution at a final concentration of 100 μM. Puffer electrodes were placed ~30 μm from the cell. Voltage commands were applied, current measurements were stored, and pressure application (3 psi) of ACh from the puffer electrodes was controlled using an Atari computer-based acquisition system and software (Instrutech, Elmont, NY). Electronic compensation was used to reduce the effective series resistance and the time constant of membrane charging, and to provide measurements of access resistance and cell membrane capacitance. Series resistance compensation of 50–70% was routinely used and reduced estimated series resistance errors to 5 mV or less. To record ACh-induced currents, the cell membrane potential was clamped to −80 mV, and the computer driven system simultaneously applied a 5 sec pressure pulse to the back of the puffer electrode while recording the current responses from the patch clamp electrode. For measurements of Na current, cells were held at −80 mV and every 3 sec the membrane potential stepped through a 40 msec prepulse to −120 mV, followed by a 20 msec depolarizing test pulse to a potential between −60 and +30 mV. Linear leakage currents and capacity transients were subtracted with scaled pulse (P/4) routines. Current signals were low-pass filtered at 10 kHz, digitized at 20 kHz for storage, and digitally filtered at 2 kHz during analysis. For both the Na current and the ACh-induced current, the maximum current elicited, along with the measurement of cell membrane capacitance, was used to calculate current density. Values given in the text are mean ± SEM. Statistical significance was determined using a two-tailed Student's *t* test.

RNA Isolation and Analysis

Cells plated at ~10^6 cells per 100 mm tissue culture dish were harvested and total cellular RNA isolated by the method of Chirgwin et al. (1979). RNAse protection analysis was performed essentially as described by Hod (1992). The cRNA probes specific for cyclophilin and the Na channel subtypes were generated using [^{32}P]UTP (New England Nuclear, Boston, MA). RNA samples (20 μg) were hybridized with 2.5×10^5 cpm of cRNA probe as described in our previous studies (Fanger et al., 1993), with the exception that the samples were digested with RNAse for 1 hr at 30°C before being separated on a 6% acrylamide gel and exposed to Kodak XAR film for 24 hr at −80°C. For Northern blot analysis, 30 μg RNA samples were size-fractionated on 0.8% agarose gels containing 2.0 M formaldehyde, transferred to a nylon membrane (Zetabind; Cuno, Meridan, CT) overnight, and the membrane baked at 80°C for 2 hr. [^{32}P]UTP-labelled cRNA probes specific for Na channel mRNA and cyclophilin mRNA were generated and hybridized with the membrane at a final activity of 6×10^6 cpm/ml and 5×10^5 cpm/ml, respectively, as described in our previous studies (Ginty et al., 1992; Fanger et al., 1993). After hybridization for 36 hr at 65°C, the membrane was washed and exposed to Kodak XAR film at −80°C for 24 hr. For analysis of nAChR subunit mRNAs, Northern blot hybridization was performed as described in our earlier studies (Henderson et al., 1994). Random-primed cDNA probes specific for nAChR subunit mRNAs were generated using a commercially avail-

able kit (Bethesda Research Laboratories, Grand Island, NY), [^{32}P]-labeled dCTP (NEN), and nAChR cDNAs. The [^{32}P]UTP-labelled cRNA probe specific for cyclophilin mRNA was generated as in our earlier studies (Henderson et al., 1994). Probes were added to the hybridization solution at a final activity of 10^6 cpm/ml. After hybridization for 14 hr at 42°C, the membrane was washed and exposed to Kodak XAR film at −80°C for 7 to 72 hr. The NIH IMAGE@ program was used for densitometric analysis of the autoradiographic signals representing the Na channel α subunit, cyclophilin, and nAChR subunit mRNAs.

Transfections and CAT Assays

Plasmid DNA for transfection was isolated by the Triton-lysozyme method (Holmes & Quigley, 1981) and purified by CsCl gradient centrifugation. Cells plated at 2×10^6 cells per 100 mm culture dish were transfected with 8 μg of plasmid DNA containing either the chloramphenicol acetyltransferase (CAT) gene under control of the promoter and 5′ flanking region of the type II Na channel α subunit gene (Maue et al., 1990), or the CAT gene under the control of the Rous sarcoma virus (RSV) promoter sequences. In addition, 2 μg of a plasmid containing the β-galactosidase (βGAL) gene under the control of the RSV promoter was included to normalize for transfection efficiency. Plasmid DNA was mixed with Opti-MEM I media (GIBCO) containing Lipofectamine (GIBCO), and the mixture added to cells rinsed in Opti-MEMI. After 4 hr, the mixture was removed and the cells rinsed in Opti-MEM I before being returned to their regular culture media. After 48 hr, cells were washed and harvested in PBS before they were lysed in 150 μl of 0.25 M Tris-HCl and 1 mM EDTA, pH 7.6, by freeze/thawing twice. Protein concentrations in the lysates were determined by the method of Bradford (1976) using a commercially available protein assay reagent (Pierce Chemical, Rockford, IL) and BSA as a standard. Lysates were assayed for βGAL activity as previously described (Norton & Coffin, 1985), with the conversion of the substrate o-nitrophenyl β-D-galactopyranoside (Sigma) quantitated spectrophotometrically. CAT activity was assayed as in our previous studies (Maue et al., 1990). Lysates were incubated at 65°C for 10 min to minimize deacylation activity (Crabb & Dixon, 1987), and then a 180 μl mixture containing 10 μg of protein, 0.4 mM acetyl coenzyme A (Sigma), and 0.1 μCi of [dichloroacetyl-1,2-^{14}C]chloramphenicol (NEN) was incubated at 37°C for 4 hr. Under these conditions, the percentage of chloramphenicol that was acetylated was between 1 and 50%. After extraction with ethyl acetate, the radioactive forms of chloramphenicol were resolved by thin layer chromatography (TLC), localized by exposing the TLC plates to X-ray film at −80°C for 24 hr, and then quantitated by liquid scintillation counting.

Results

Altered Na Current Density and nAChR Current Density in nnr Variants of the PC12 Cell Line

There is a striking difference between functional Na channel expression in the nnr variants of the PC12 cell line that we analyzed and that typically observed in wild-type PC12 cells. In whole-cell patch-clamp recordings from wild-type PC12 cells, little or no Na current can usually be elicited from the untreated cells, while in response to NGF there are large, easily detectable Na currents (Fig. 1*A*). In contrast, in the nnr5 PC12 variant,

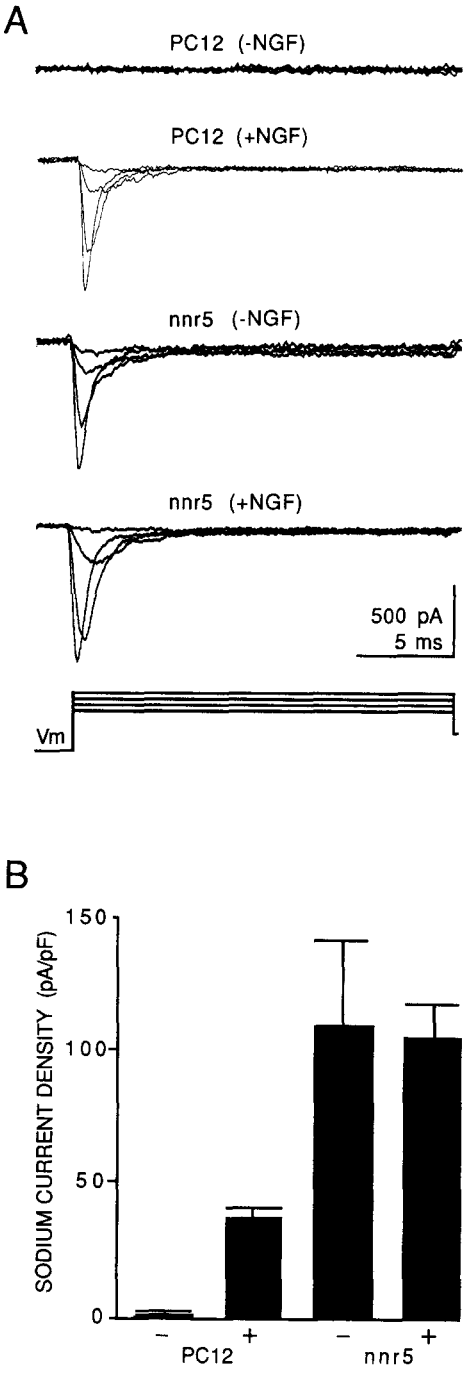

Fig. 1. Functional Na channel expression in wild-type and nnr5 variants of PC12 cells maintained in the absence and presence of NGF. Shown in *A* are representative recordings of Na currents in wild-type (above) and nnr5 variants (below) of PC12 cells that were either untreated or treated with 100 ng/ml NGF for 7 days. Superimposed current records illustrate the response to depolarizing pulses to −40, −30, −20, and −10 mV, from a prepulse potential of −120 mV and holding potential of −80 mV. Plotted in *B* is the average Na current density in untreated (−) and NGF-treated (+) wild-type PC12 cells and nnr5 cells. Error bars represent the SEM. Cell membrane capacitance (mean ± SEM; pF) for untreated and NGF-treated cells, respectively, was 6.5 ± 0.2 (n = 30) and 14.8 ± 0.9 (n = 32) for wild-type PC12 cells; 6.4 ± 0.6 (n = 12) and 6.1 ± 0.6 (n = 13) for nnr5 cells.

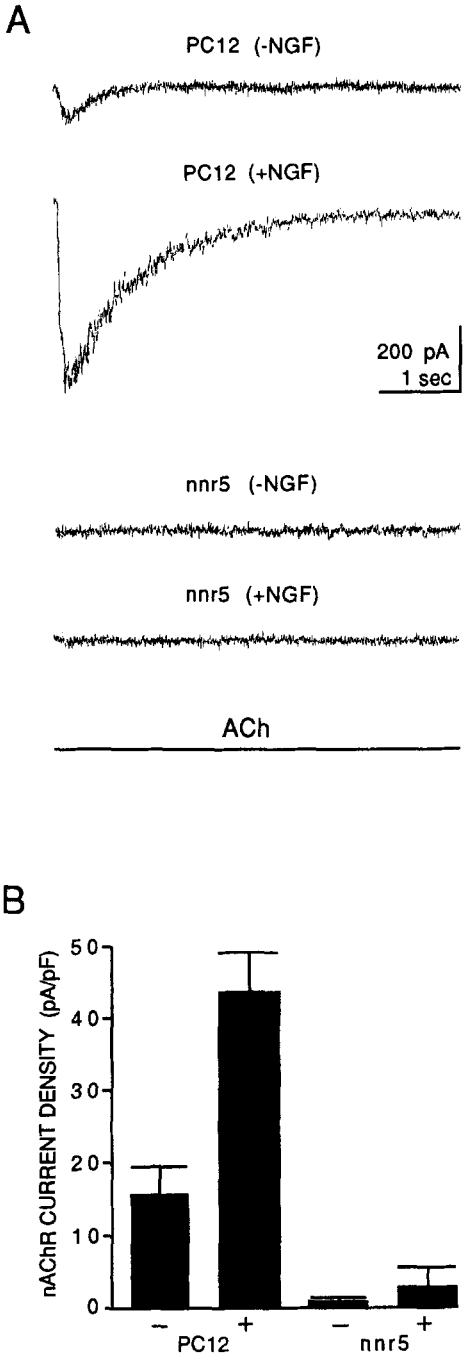

Fig. 2. Functional nAChR channel expression in wild-type and nnr5 variants of PC12 cells maintained in the absence and presence of NGF. Shown in *A* are representative recordings of ACh-induced currents in wild-type (above) and nnr5 variants (below) of PC12 cells that were either untreated or treated with 100 ng/ml NGF for 7 days. The duration of the pressure application of ACh from the puffer electrode is indicated by the solid bar below the current records. Plotted in *B* is the average ACh-induced current density in untreated (−) and NGF-treated (+) wild-type PC12 cells and nnr5 cells. Error bars represent the SEM. Cell membrane capacitance (mean ± SEM; pF) for untreated and NGF-treated cells, respectively, was 6.5 ± 0.2 (n = 36) and 14.5 ± 0.8 (n = 39) for wild-type PC12 cells; 5.7 ± 0.5 (n = 15) and 5.9 ± 0.8 (n = 10) for nnr5 cells.

large Na currents can be elicited in both untreated and NGF-treated cells, with NGF failing to increase the magnitude of the Na currents (Fig. 1*A*). When combined with the fact that NGF does not cause an increase in the size of the nnr5 cells as it does to the wild-type PC12 cells (as indicated by cell membrane capacitance), there is a significant difference in the Na current density in the two cell types, with the Na current density in both untreated (n = 12) and NGF-treated (n = 13) nnr5 cells significantly ($P < 0.01$) greater than observed in wild-type PC12 cells treated with NGF (Fig. 1*B*). Large Na currents and elevated Na current densities were also characteristic of "recloned" nnr5 cells generated from a single cell of the original nnr5 subline (n = 10) as well as nnr3 cells (n = 28) (*data not shown*), the latter a separate trk-deficient PC12 subline isolated during the same mutagenesis and selection as the nnr5 subline (Green et al., 1986).

The expression of functional nAChR channels in the nnr variants also contrasts with that observed in wild-type PC12 cells. In wild-type PC12 cells, most untreated cells (29/36) exhibit small, yet detectable (>25 pA) inward currents in response to ACh application (Fig. 2*A*). Upon treatment of the cells with NGF, the currents are generally much larger (Fig. 2*A*), with both the average ACh-induced current and the nAChR current density in NGF-treated PC12 cells (n = 39) significantly ($P < 0.01$) greater than in untreated cells (n = 36) (Fig. 2*B*). In contrast, the response of the nnr variants to ACh was markedly reduced in both untreated and NGF-treated cells (Fig. 2*A*). The proportion of untreated nnr5 cells that exhibited inward currents in response to ACh (2/15) was significantly smaller ($P < 0.05$) than for untreated wild-type PC12 cells. In the few cells that did respond, the ACh-induced currents were barely detectable (20–30 pA), so that both the average ACh-induced current and the nAChR current density in the untreated nnr5 cells were also significantly lower ($P < 0.01$) than in the untreated wild-type cells. In response to NGF treatment there was no significant change in the extent of functional nAChR expression in the nnr5 cells. The proportion of NGF-treated nnr5 cells exhibiting ACh-induced inward currents (1/10), the average ACh-induced current, and the nAChR current density (Fig. 2*B*) were again all significantly smaller ($P < 0.05$) than observed for untreated, much less NGF-treated, wild-type PC12 cells. The reduced expression of functional nAChR in the NGF-treated nnr5 cells was also evident in outside-out patches of membrane excised from these cells. Whereas ACh-induced single channel activity occurs in more than half (62%) of the membrane patches isolated from NGF-treated wild-type PC12 cells (Henderson et al., 1994), no ACh-induced activity was detected in the outside-out patches of membrane isolated from NGF-treated nnr5 cells (n = 5). Similar results were also obtained with nnr3 cells, another trk-deficient PC12 variant. In both

untreated and NGF-treated nnr3 cells (n = 28), the proportion of the cells with ACh-induced currents, the average ACh-induced current, and the nAChR current density were all significantly lower ($P < 0.01$) than observed in untreated wild-type PC12 cells (*data not shown*).

Expression of Na Channel Genes and nAChR Subunit Genes in nnr Variants

The expression of RNA transcripts encoding Na channel and nAChR subunits in the nnr cells differs from that observed in wild-type PC12 cells, and may provide a basis for the altered expression of functional ion channels in these cells. For analysis of Na channel α subunit mRNA, Northern blot hybridization was carried out using a probe corresponding to a region highly conserved among Na channel α subunit genes (*see* Materials and Methods). As an internal control for variations in RNA loading, the Na channel mRNA signals were normalized to the signal representing cyclophilin mRNA, which is constitutively expressed in PC12 cells and unaffected by growth factor treatment (Machida et al., 1989). As we have shown previously (Ginty et al., 1992; Fanger et al., 1993), in wild-type PC12 cells there are low levels of Na channel α subunit mRNA in the untreated cells, with a severalfold increase in the steady state levels of Na channel α subunit mRNA occurring in response to NGF (Fig. 3*A*). In marked contrast to the wild-type PC12 cells, there are constitutively elevated levels of Na channel α subunit mRNA in the untreated nnr5 cells (Fig. 3*A*). Although NGF did not cause any further increase, the levels of Na channel α subunit mRNA in both the untreated and NGF-treated nnr5 cells were considerably higher than in NGF-treated wild-type cells (Fig. 3*A*), paralleling the elevated expression of functional Na channels in the nnr5 cells. To determine whether expression of any of the identified Na channel α subunit genes expressed in the nervous system could account for the elevated Na channel mRNA in the nnr5 cells, RNAse protection assays were carried out using probes specific for the type I, II, and III Na channel α subunit mRNAs (Fanger et al., 1993). Consistent with results from wild-type PC12 cells, type II Na channel mRNA was detected in RNA samples from untreated and NGF-treated nnr5 cells (Fig. 3*B*), while type I and type III Na channel α subunit mRNAs were not detected (*data not shown*). However, in these assays the level of type II Na channel α subunit mRNA in the nnr5 cells did not appear to be substantially elevated in comparison to the levels found in wild-type PC12 cells (Fig. 3*B*). Consistent with this, when a fusion gene consisting of the chloramphenicol acetyltransferase gene under the control of the 5′ flanking region of the type II Na channel α subunit gene (Maue et al., 1990) was transfected into wild-type and nnr5 PC12 cells, there was no difference in the level of

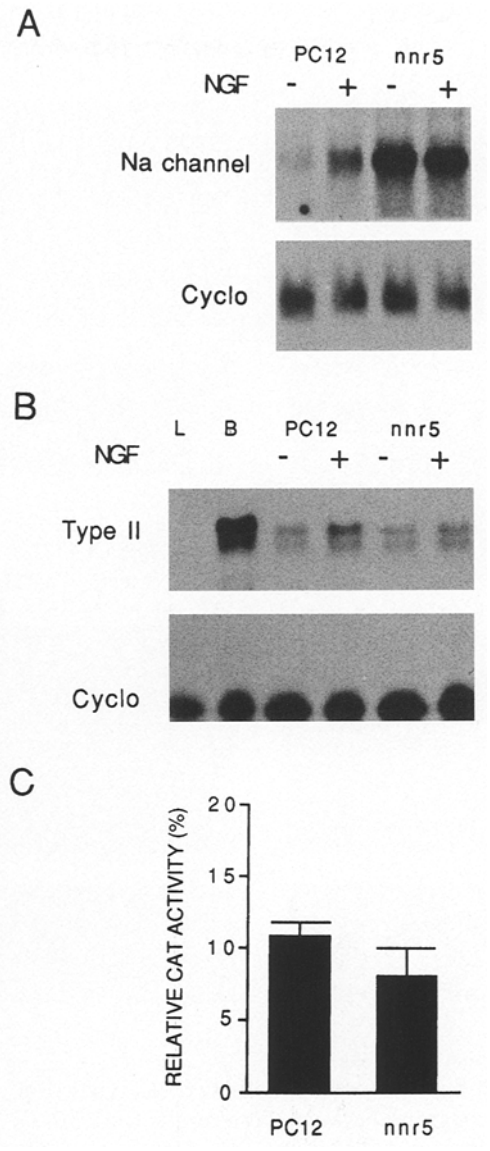

Fig. 3. Na channel α subunit gene expression in wild-type PC12 cells and the nnr5 variant of PC12 cells. Samples of RNA from wild-type and nnr5 cells that were either untreated (−) or treated with 100 ng/ml NGF for 7 days (+) were analyzed using (*A*) Northern blot hybridization (40 μg samples) and (*B*) RNAse protection assays (20 μg samples). For the Northern analysis, a probe capable of recognizing many forms of Na channel α subunit mRNA was used, while in the RNase protection assays a probe specific for type II Na channel α subunit mRNA was used (*see* Materials and Methods). In both assays, a probe specific for cyclophilin mRNA was also included and the signals representing cyclophilin mRNA used as an internal control for variations in RNA loading. In *A* and *B*, representative experiments are shown, with the signals representing Na channel mRNA (Na channel; Type II) shown after 24 hr exposure to film, and the signals representing cyclophilin mRNA (Cyclo) shown after 30 min exposure to film. In *B*, mRNA samples from liver (*L*) and brain (*B*) were also included. Plotted in *C* is the relative expression of a type II Na channel-CAT fusion gene in wild-type PC12 cells and nnr5 cells. The average from four experiments is shown, with Na channel-CAT fusion gene activity expressed as a percentage of the activity of a constitutively expressed Rous Sarcoma Virus-CAT fusion gene in that cell type. Error bars represent the SEM.

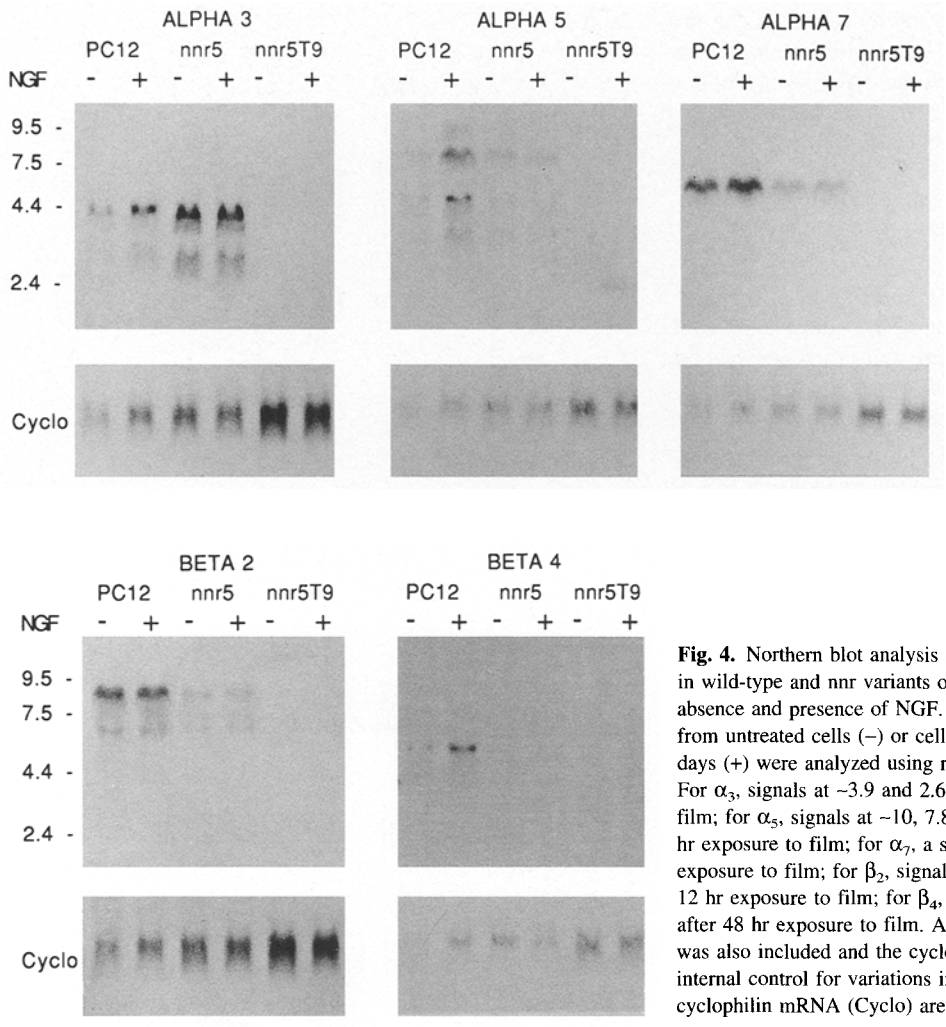

Fig. 4. Northern blot analysis of nAChR subunit mRNA expression in wild-type and nnr variants of PC12 cells maintained in the absence and presence of NGF. In each blot, 30 μg samples of RNA from untreated cells (–) or cells treated with 100 ng/ml NGF for 7 days (+) were analyzed using random-primed, ^{32}P-labeled probes. For α_3, signals at ~3.9 and 2.6 kb are shown after 4 hr exposure to film; for α_5, signals at ~10, 7.8, 4.5, and 2.8 kb are shown after 48 hr exposure to film; for α_7, a signal at ~5.5 kb is shown after 12 hr exposure to film; for β_2, signals at ~7.6 and ~5.3 kb are shown after 12 hr exposure to film; for β_4, signals at ~5.1 and 3.5 kb are shown after 48 hr exposure to film. A probe specific for cyclophilin mRNA was also included and the cyclophilin mRNA signal used as an internal control for variations in RNA loading. Signals representing cyclophilin mRNA (Cyclo) are shown after 30 min exposure to film.

expression of this fusion gene in the two cell types (Fig. 3*C*). The incongruity between these results (Fig. 3*B, C*) and the elevated levels of Na channel mRNA detected in nnr5 cells by Northern blot analysis (Fig. 3*A*) suggests that type II Na channel α subunit mRNA, although contributing to the elevated level of Na channel α subunit mRNA in the nnr5 cells, may not account for all of the Na channel α subunit mRNA detected in nnr5 cells by Northern blot analysis.

The expression of RNA transcripts encoding nAChR subunits in wild-type and nnr5 PC12 cells was also compared, using cDNA probes and Northern blot hybridization conditions which allow subunit-specific mRNA transcripts to be detected (*see* Materials and Methods). The signals representing the individual nAChR mRNAs were normalized to the signal representing the constitutively expressed cyclophilin mRNA. In wild-type PC12 cells, RNA transcripts corresponding to the α_3, α_5, α_7, β_2, and β_4 nAChR subunits were detected in untreated cells (Fig. 4), with multiple transcripts evident for the α_3, α_5, β_2, and β_4 subunits, consistent with previous reports (Boulter et al., 1986, 1990; Rogers et al., 1992; Henderson et al., 1994). In response to NGF, the steady-state level of these transcripts increased 1.5 to 2.5-fold, as we have previously shown (Henderson et al., 1994). In comparison, the expression of nAChR subunit mRNAs in the nnr5 cells was noticeably different (Fig. 4). Among the most notable differences was that the level of expression of α_3 transcripts in untreated nnr5 cells was much higher than the level detected even in NGF-treated, much less untreated, wild-type PC12 cells (Fig. 4). Not all of the nAChR subunit mRNAs were elevated in nnr5 cells, as the level of α_5 mRNA in these cells was comparable to the level expressed in untreated wild-type PC12 cells, and the expression of α_7 mRNA, although detectable, was actually much lower than in untreated wild-type PC12 cells (Fig. 4). Also striking was the apparent lack of β_4 transcripts in the nnr5 cells as well as a substantially reduced level of β_2 mRNA in comparison to wild type PC12 cells (Fig. 4). Finally, in contrast to wild-type PC12 cells, NGF had no effect on the expression of nAChR transcripts in the nnr5 subline (Fig. 4).

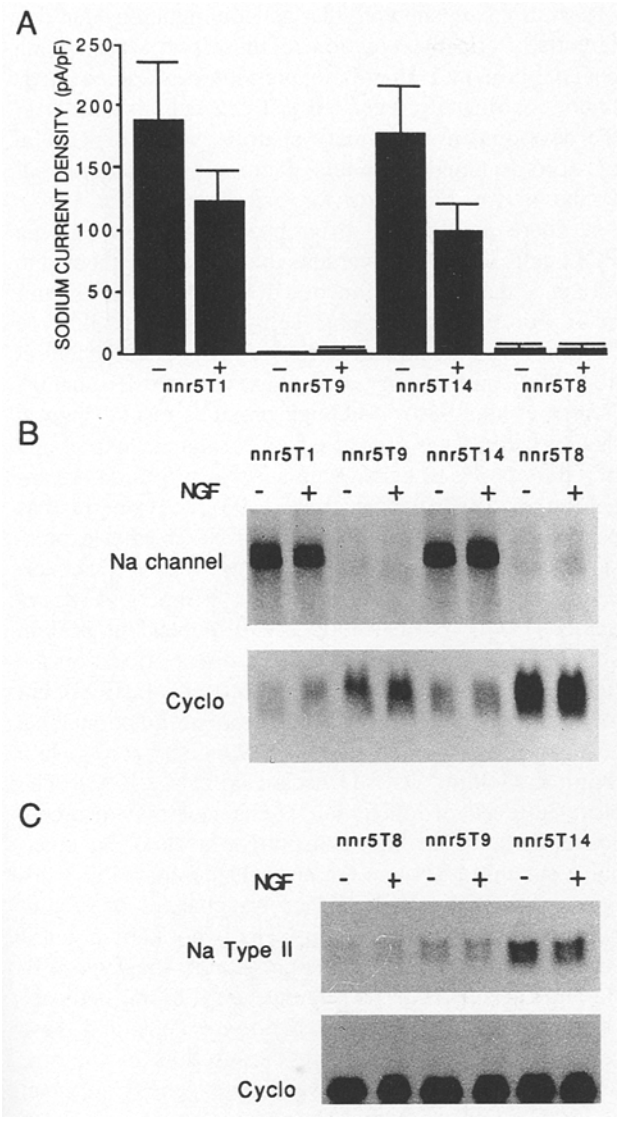

Fig. 5. Na channel current density and α subunit mRNA expression in nnr cells stably transfected with a cDNA encoding trkA. Shown in *A* is the average Na current density in untreated (–) and NGF-treated (+) cells that have been transfected with the trkA cDNA and either do not express trkA (nnr5T1), overexpress trkA, but do not exhibit tyrosine phosphorylated trkA in the absence of NGF (nnr5T9, nnr5T14), or overexpress trkA and exhibit a low level of tyrosine phosphorylated trkA in the absence of NGF (nnr5T8). Cell membrane capacitance (mean ± SEM; pF) for untreated and NGF-treated cells, respectively, was 4.8 ± 0.5 (n = 5) and 6.4 ± 0.4 (n = 10) for nnr5T1 cells; 15.5 ± 1.9 (n = 20) and 18.8 ± 1.6 (n = 20) for nnr5T9 cells, 4.2 ± 0.4 (n = 5) and 16.8 ± 3.3 (n = 10) for nnr5T14 cells, and 10.9 ± 0.9 (n = 25) and 15.4 ± 1.7 (n = 14) for nnr5T8 cells. Shown in *B* is Northern blot analysis of Na channel mRNA in nnr5T1, nnr5T9, nnr5T14, and nnr5T8 cells that were either untreated (–) or treated with 100 ng/ml NGF for 7 days (+). The probe used is capable of recognizing many forms of Na channel α subunit mRNA (*see* Materials and Methods). A probe specific for cyclophilin mRNA was also included and the signals representing cyclophilin mRNA (Cyclo) used as an internal control for variations in RNA loading. In *C*, RNAse protection analysis of 20 μg samples of RNA from nnr5T8, nnr5T9, and nnr5T14 variants of PC12 cells maintained in the absence (–) or presence (+) of NGF for 7 days. Probes specific for type II Na channel mRNA and cyclophilin mRNA were used. In *B* and *C*, representative experiments are shown, with signals representing Na channel mRNA (Na channel; Na Type II) shown after 24 hr exposure to film, and cyclophilin signals (Cyclo) shown after 30 min exposure to film.

Ion Channel Expression in nnr Cells Stably Transfected with a cDNA Encoding trkA

It has been shown that transfection of nnr5 cells with a cDNA encoding a trkA neurotrophin receptor restores many of the NGF-dependent responses normally observed in PC12 cells. Specifically, in nnr sublines stably transfected with trkA, the binding and internalization of NGF occurs with characteristics similar to wild-type cells, and NGF treatment leads to increased tyrosine phosphorylation, cellular hypertrophy, survival in serum-free medium, the induction of immediate early genes such as c-fos, c-jun, and NGFIB, the induction of genes associated with neuronal phenotype, such as transin, peripherin, and NILE, as well as neurite outgrowth and regeneration (Loeb & Greene, 1993). To determine whether expression of trkA would influence Na channel and nAChR expression in these cells when maintained either in the absence or presence of NGF, whole-cell patch clamp recordings, Northern blot analysis and RNase protection assays were used to analyze these previously established nnr sublines. The nnr5T8, nnr5T9, and nnr5T14 sublines all overexpress trkA mRNA and trkA protein compared to wild-type PC12 cells, and exhibit NGF-dependent tyrosine phosphorylation of trkA (Loeb & Greene, 1993). In addition, in the nnr5T8 cells there is also a basal level of phosphorylated (and presumably active) trkA (Loeb & Greene, 1993). Analysis of the nnr5T1 subline, which is resistant to neomycin, yet does not express trkA nor extend neurites in response to NGF, served as a control.

As might be expected, Na channel expression in the control nnr5T1 subline was similar to that observed in the parental nnr5 cells, providing an indication that the

transfection and selection procedure itself did not interfere with Na channel expression. There were large Na currents and elevated Na current densities in these cells (Fig. 5*A*), with slightly lower, though not significantly different, levels of expression in nnr5T1 cells treated with NGF for 7 days. As in the parental nnr5 cells, the elevated expression of functional Na channels was accompanied by elevated levels of Na channel α subunit mRNA (Fig. 5*B*). In the nnr5T9, nnr5T14, and nnr5T8 sublines, there did not appear to be any consistent effect of trkA expression on the expression of functional Na channels or Na channel α subunit mRNA (Fig. 5). In the nnr5T8 and nnr5T9 sublines, the density of functional Na channels was very low, even when maintained for 7 days in the presence of NGF (Fig. 5*A*). This was consistent with the almost complete absence of Na channel α subunit mRNA in these cells, irrespective of whether they were maintained in the presence or absence of NGF (Fig. 5*B, C*). In contrast, there were large Na currents, elevated Na current densities, and elevated levels of Na channel α subunit mRNA in the nnr5T14 subline, even in the absence of NGF (Fig. 5).

The expression of trkA did not influence the expression of functional nAChR nor nAChR subunit mRNAs in the nnr sublines. No ACh-induced currents were detected in whole-cell recordings from nnr5T1 (0/10), nnr5T8 (0/22), nnr5T9 (0/11), or nnr5T14 (0/25) cells maintained in the presence or the absence of NGF for 7 days. This lack of functional responses was consistent with our inability to detect any of the nAChR subunit mRNAs in untreated or NGF-treated nnr5T9 cells using Northern blot analysis (Fig. 4).

Discussion

We have analyzed voltage-dependent Na channel and nAChR expression in genetic variants of the PC12 cell line using electrophysiological and molecular biological approaches, and find the expression of these two channel proteins substantially altered in comparison to that typically observed in wild-type PC12 cells. In the nnr variants, the levels of both Na channel mRNA and functional Na channels are dramatically elevated in comparison to the wild-type cells. In stark contrast, the levels of specific nAChR subunit mRNAs are unusually low and the expression of functional nAChR channels barely detectable in these cells. This was observed in multiple nnr subclones, including nnr5 cells "recloned" from a single cell of the original nnr5 subline, and persisted throughout multiple passages in cell culture. The dissimilar expression of Na channels and nAChR in the nnr variants suggests the mechanisms governing their expression are at least partially distinct, consistent with earlier evidence in wild-type PC12 cells (Ifune & Steinbach, 1990; Ginty et al., 1992, Henderson et al., 1994), and extends those observations by showing that genetic mutations can differentially affect expression of these two types of ion channel proteins. That the expression of these channels is not coordinately regulated in PC12 cells is similar to the development of primary neurons, where expression of voltage-gated channels occurs independently of ligand-activated channels (for review, *see* Spitzer, 1985).

There are known differences between wild-type PC12 cells and the nnr variants that might be expected to influence the levels of functional Na channels in the nnr cells. For example, the nnr5 cells differ from wild-type PC12 cells in having high levels of ras-GTP (Stephens et al., 1994) and enhanced responses to cAMP analogs (Green et al., 1986). Although previous results suggest that ras activity has little effect on Na channel expression in wild-type PC12 cells (Kalman et al., 1990; D'Arcangelo et al., 1993; Fanger et al., 1993), activated ras has been shown to influence functional Na channel expression in a variety of cell types (Caffrey, Brown & Schneider, 1987; Estacion, 1990; Flamm, Birnberg & Kaczmarek, 1990). Furthermore, cAMP-dependent protein kinase is not only thought to have direct effects on the functional properties of Na channels (Li et al., 1992), but to influence the level of expression of functional Na channels as well (Kalman et al., 1990; Ginty et al., 1992; Smith & Goldin, 1992; D'Arcangelo et al., 1993). The elevated levels of functional Na channels may also be a consequence of the high constitutive levels of Na channel α subunit mRNA in the nnr cells compared to wild-type cells. Why the levels of Na channel α subunit mRNA are elevated in the nnr cells is not known, but it is intriguing, given that the expression of the Type I, II, IIa and III Na channel α subunit genes in nnr cells appears to be similar to that in wild-type cells, and these cells do not appear to express transcripts of the size reported for a glial-specific Na channel gene (Gautron et al., 1992). Other putative Na channel genes have been reported (D'Arcangelo et al., 1993), and it will be of interest in future studies to identify the Na channel α subunit genes expressed in nnr cells in addition to the Type II gene, as well as to determine if differences in transcriptional activity, mRNA stability, or both, account for the elevated levels of Na channel mRNA in these cells. In addition, these cells may prove useful for identifying factors that influence Na channel gene expression and, because of the high constitutive levels of functional channels, provide a convenient system for analysis of Na channel function.

The response of nnr cells to ACh also contrasts with that typically observed in wild-type PC12 cells, although unlike the Na currents, the responses to ACh were rarely observed and barely detectable. While the reason for the diminished response to ACh is unknown, reduced expression of specific nAChR subunit mRNAs may provide an explanation. In primary neurons expressing α_3, α_5, α_7, β_4, and possibly β_2, subunits, data indicate that

transcript levels may be rate limiting in the accumulation of nAChRs (Corriveau & Berg, 1993). In nnr cells, there is a striking decrease in the expression of β subunit mRNAs compared to wild-type PC12 cells, with no evidence of β_4 mRNA and β_2 mRNA barely detectable. Coupled with studies demonstrating that coexpression of β subunits with α subunits is often required to produce functional nAChR channels (Boulter et al., 1987; for reviews, *see* Luetje, Patrick & Seguela, 1990; Deneris et al., 1991), the decreased levels of β subunit mRNAs in nnr cells could result in a dramatic decrease in the expression of functional nAChR. Specifically, although α_3 mRNA levels are extremely high in nnr cells, the lack of an accompanying β subunit, which appears to be required for formation of a functional channel from α_3 subunits (Boulter et al., 1987), may severely limit the formation of functional channels in these cells. For although α_7 subunits can form functional channels in the absence of β subunits (Seguela et al., 1993), this transcript is expressed at very low levels in nnr cells, and α_5 subunit mRNA, although expressed at comparable levels in nnr and wild-type PC12 cells, encodes a subunit that does not appear to form functional channels by itself or with other subunits (for review, *see* Luetje et al., 1990). The altered expression of nAChR transcripts is also interesting with regard to regulation of the nAChR subunit gene family. The strikingly elevated level of α_3 mRNA, in the absence of β_4 mRNA and presence of relatively modest levels of α_5 mRNA, illustrates that the α_3, α_5, and β_4 genes, though arranged in a gene cluster (Boulter et al., 1990), can be independently regulated within the same cell. It also suggests there is a mechanism by which α_3 mRNA levels can be selectively enhanced, and invites further analysis of α_3 expression in these cells.

The nnr variants of the PC12 cell line have allowed rapid progress to be made in understanding many of the early events associated with the activation of the trk receptor (Altin et al., 1991; Loeb et al., 1991; Loeb & Greene, 1993; Loeb et al., 1994; Stephens et al., 1994). While some of the later changes in gene expression induced by NGF have also been "rescued" upon trk expression in the nnr cells (Loeb et al., 1993), it is intriguing that other late responses are not. For example, thymosin β_4 mRNA is expressed in wild-type PC12 cells and increases in response to NGF (Leonard, Ziff & Greene, 1987). However, it is not expressed in nnr cells, nor is it induced by NGF when trk is introduced into the cells (Loeb et al., 1993). Similarly, in nnr cells, there are apparently influences on Na channel and nAChR expression that override the regulation normally exerted by trk receptor activation. Given the suggestion that a single or very small number of genetic changes distinguish wild-type PC12 cells from nnr cells (Green et al., 1986), nnr cells may provide a valuable means to identifying molecular elements governing the differential expression of these two ion channel proteins, as well as key points of divergence in the signalling pathways underlying the effects of growth factors on gene expression and neuronal differentiation.

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