

# The Developmental Increase in ACh Current Densities on Rat Sympathetic Neurons Correlates with Changes in Nicotinic ACh Receptor $\alpha$ -Subunit Gene Expression and Occurs Independent of Innervation

A. Mandelzys,<sup>1</sup> B. Pié,<sup>1</sup> E. S. Deneris,<sup>2</sup> and E. Cooper<sup>1</sup>

<sup>1</sup>Department of Physiology, McGill University, Montréal, Québec, Canada H3G 1Y6 and <sup>2</sup>Department of Neuroscience, Case Western Reserve University, Cleveland, Ohio 44106

**Determining factors that control the expression of neurotransmitter receptors and the mechanisms by which these factors operate is essential to understand how synapses form during development and how receptor numbers change in the adult. In this study, we have investigated one such factor, the influence of innervation, on the developmental expression of nicotinic ACh receptors (nAChRs) on neonatal rat sympathetic neurons, both in terms of ACh current densities, and in terms of mRNA levels for the transcripts that encode these receptors. To date, nine genes have been cloned that encode neuronal nAChR subunits in mammals. We demonstrate that mRNA encoding five nAChR subunits,  $\alpha 3$ ,  $\alpha 5$ ,  $\alpha 7$ ,  $\beta 2$ , and  $\beta 4$ , are present in neonatal rat sympathetic neurons. We show that mRNA levels for  $\alpha 3$  and  $\alpha 7$  subunits increase by more than threefold over the first 2 postnatal weeks, a period when most synapses are forming on the neurons; however, we observed no significant change in mRNA levels for  $\alpha 5$ ,  $\beta 2$ , or  $\beta 4$ . Using whole-cell voltage-clamp techniques, we show that the increase in  $\alpha$ -subunit mRNA correlates with increases in ACh current densities, which double over the same period. To investigate the role of innervation, we cut the preganglionic nerve at birth and measured subunit mRNA levels and ACh current densities in denervated neurons 1–2 weeks later. Our results indicate that the preganglionic nerve differentially affects the mRNA level for the five nAChR transcripts, yet it has little influence on the developmental increase in ACh current densities.**

**[Key words: nicotinic receptors, neuronal ACh receptors, ligand-gated ion channels, sympathetic neurons, gene expression, RNase protection]**

Studies on the neuromuscular junction have elucidated many molecular details involved in forming chemical synapses, and have generated some governing principles for synaptogenesis. These studies have shown that as synapses form, several com-

plex processes coordinate the expression of various proteins both in the presynaptic motor nerve terminals and in the postsynaptic muscle membranes (Hall and Sanes, 1993). Presumably, most of these processes also occur when synapses form between neurons; however, much less is known about the development of neuron–neuron synapses, and consequently it has not been fully resolved which of the principles that govern synaptogenesis on muscle also hold true for synapse formation on neurons. Given the dissimilarities between neurons and muscle as postsynaptic targets, one might expect that some discrepancies exist.

To learn more, we have investigated one aspect of synapse formation on neurons: the influence of the presynaptic nerve on mRNA levels for neurotransmitter receptor subunits during early postnatal development, and associated changes in the functional expression of these receptors. In mammalian muscle, embryonic myoblasts initially express all four nicotinic ACh receptor (nAChR) subunits,  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ , shortly after fusion (reviewed by Hall and Sanes, 1993). However, in the early postnatal period, as the motor nerve terminals are establishing synaptic contact with muscle, the nuclei near the site of contact increase mRNA production for  $\alpha$ ,  $\beta$ , and  $\delta$  subunits, and they initiate expression of the  $\epsilon$  subunit *de novo*; as  $\epsilon$  mRNA increases,  $\gamma$  mRNA production declines (Witzemann et al., 1989; Brenner et al., 1990; Martinou and Merlie, 1991). Nuclei away from the synapse continue to produce mRNA for  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ , but at much lower levels than those near the synapse (Witzemann et al., 1989; Brenner et al., 1990). This changing pattern of expression for nAChR subunits in muscle, together with other factors, leads to a high density of  $\alpha_2\beta\epsilon\delta$  receptors at the synapse, and a low density of  $\alpha_2\beta\gamma\delta$  receptors elsewhere on the muscle membrane (Mishina et al., 1986; Gu and Hall, 1988).

In this article, we ask whether neurons undergo similar changes in expression of mRNA for neurotransmitter receptor subunits during early postnatal development as synapses form, and if so, what role do the presynaptic terminals have in this process. For our experiments, we have focused on the expression of nAChRs on neonatal rat sympathetic neurons, a preparation that has served as a model to investigate many aspects of synapse formation among neurons (Purves and Lichtman, 1985). In fact, most of what is known about the developmental expression of neuronal nAChRs has come from studies on autonomic ganglia (Schuetze and Role, 1987; Berg et al., 1989; Sargent, 1993). One advantage of this preparation, in addition to its easy accessibility, is that in the rat, most of the synapses on sympathetic

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Correspondence should be addressed to Dr. E. Cooper, Department of Physiology, McIntyre Medical Sciences Building, McGill University, 3655 Drummond Street, Montréal, Québec, Canada H3G 1Y6.

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neurons form over the first few postnatal weeks: the number of synapses at birth is only about 10% of that on sympathetic neurons in adult rats, and this number increases to about 90% over the next 3 weeks (Smolen and Raisman, 1980; Schafer et al., 1983; Rubin, 1985); this has allowed us to investigate changes in postnatal neurons over a period when a large number of synapses are being formed.

## Materials and Methods

**RNAse protection assay.** Total cellular RNA was isolated from superior cervical ganglia (SCG) dissected from postnatal day 1 (P1), P7, and P14 rats (CD strain, Charles River Canada), and from P7 and P14 rats in which the preganglionic nerve to the SCG on one side had been cut. The RNA was extracted from the ganglia with a guanidinium isothiocyanate-phenol-chloroform extraction procedure described by Chomczynski and Sacchi (1987). In some experiments, in order to isolate RNA from relatively pure neuronal and non-neuronal cell populations from the ganglia, the ganglia were enzymatically dissociated (Mandelzys and Cooper, 1992) and the resultant cell suspension was centrifuged through a 35% Percoll (Pharmacia) gradient that separates the cell suspension into a neuronal fraction and non-neuronal cell fraction; this was routinely confirmed by culturing aliquots from each fraction. Total cellular RNA was then isolated from each fraction separately as above. Typically, the non-neuronal cells contribute less than 10% of the total cellular RNA from P1–P14 ganglia. After dissociation, we recover approximately 70–80% of the RNA of the intact ganglia, which is roughly the same proportion of viable neurons after this procedure. This suggests that by dissociating the ganglia we are not causing a preferential loss of non-neuronal cells, thereby underestimating their contribution. In some control experiments, total cellular RNA was isolated from the brains of P1 animals using the same procedure as above.

The RNase protection assays were according to that described by Krieg and Melton (1987). Briefly, <sup>32</sup>P-radiolabeled antisense RNA probes to  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 5$ ,  $\alpha 7$ ,  $\beta 2$ ,  $\beta 3$ , and  $\beta 4$  nAChR subunits were synthesized using an *in vitro* transcription method. Linearized plasmids, pGEM, or pSP64, containing subcloned portions of the cDNA for each neuronal nAChR subunit, were transcribed using a bacteriophage SP6, T3, or T7 RNA polymerase. The following region of each cDNA was used:  $\alpha 2$  [HYP16(9); Wada et al., 1988], bases 1737–1932, protected length 195 nucleotides;  $\alpha 3$  [pPCA48E4; Boulter et al., 1986], bases 1557–1815, protected length 258 nucleotides;  $\alpha 4$  [p3ZHYA23(2)B2; Rogers et al., 1992], bases 0–553, protected length 553 nucleotides;  $\alpha 5$  (AAlpha 5; Boulter et al., 1990), bases 1160–1639, protected length 479 nucleotides;  $\alpha 7$  [PHIP306(s)], bases 0–476, protected length 476 nucleotides;  $\beta 2$  [pGPR49; Deneris et al., 1988], bases 1617–2195, protected length 578 nucleotides;  $\beta 3$  [pESD7; Deneris et al., 1989], bases 568–1021, protected length 453 nucleotides;  $\beta 4$  [pZPC13; Duvoison et al., 1989], bases 2049–2460, protected length 411 nucleotides. The thermal stabilities for all probes, as calculated from their melting temperatures, differed by less than 1°C. Each probe was gel purified before use. For each reaction, 2–10  $\mu$ g of total cellular RNA was combined with 100,000–200,000 cpm of radiolabeled probe and allowed to hybridize overnight at 60°C. The remaining single-stranded RNAs were digested with RNase T1 and the protected RNA:RNA duplexes were denatured and run on a 5% polyacrylamide–8 M urea gel. The gels were exposed to film (Kodac XAR) at –70°C for 15–24 hr. In addition, the protected bands were quantified with a phosphor imaging system (Fujix BAS 2000, Bio Image Analyzer), or, in early experiments, by densitometry. The specific activity of each riboprobe was calculated from the number of adenine bases. To quantify relative levels of mRNA among different transcripts, the relative intensities of the hybridization signals were divided by the specific activity of the corresponding riboprobe. As a control, we performed RNase protection assays with two different  $\alpha 7$  riboprobes that protected non-overlapping regions of  $\alpha 7$  mRNA, and also differed in length. The ratio of the hybridization signal for the two probes was in close agreement with that predicted from the specific activity of each probe. In experiments to quantify changes in mRNA over the first 2 postnatal weeks, RNA for P1, P7, and P14 were always run on the same gel, as was the case for changes between control and denervated neurons.

**Preganglionic denervation.** The surgical procedures used to cut the preganglionic nerve to the SCG in P1 rats was similar to that described by Voyvodic (1987) and to that which we have used previously (McFarlane and Cooper, 1992). First, P1 rat pups were anesthetized by

cooling, and then the right sympathetic trunk was exposed and ligated midway between the ganglion and the first rib; the nerve was cut rostral to the ligature and the caudal stump displaced ventrally to impede reinnervation of the ganglion. In control experiments, we recorded from the postganglionic nerve while stimulating the preganglionic stump to confirm that we had removed the preganglionic inputs to the SCG. In addition, when the eyes opened at P12, the denervated animals had pronounced ptosis of the right eye. For all experiments involving RNase protection assays with total cellular RNA from denervated ganglia, total cellular RNA from the left SCGs with the preganglionic nerve intact served as the control. In these animals, the total cellular RNA from denervated ganglia was usually 90–100% of that in control ganglia.

**Electrophysiology.** ACh current densities were measured with whole-cell patch-clamp techniques (Hamill et al., 1981). Acutely dissociated sympathetic neurons from SCG dissected from P1 or P14 rats, in culture from 18 to 30 hr, were voltage clamped at –50 mV while 50  $\mu$ M ACh dissolved in perfusion media (see below) was applied to cell body by pressure ejection from a puffer pipette positioned 20–30  $\mu$ m from the soma; the pipettes had tip diameters of 15–20  $\mu$ m (Mandelzys and Cooper, 1992). The recordings were done with a LIST EPC-7 amplifier at room temperature (22–23°C), and the data were filtered at 3 kHz, digitized at 44 kHz by a pulse code modulating unit (PCM 701, Sony), and stored on a Beta VCR (Sony). The patch electrodes had resistances of 2–6 M $\Omega$ , and the current was balanced to zero in the perfusion solution. All currents were normalized to cell capacitance by integrating the capacity current evoked by a 5 mV hyperpolarizing voltage step from a holding potential of –50 mV. The recording chamber was continually perfused at a rate of 1 ml/min with a solution consisting of 140 mM NaCl, 5.4 mM KCl, 2.8 mM CaCl<sub>2</sub>, 0.18 mM MgCl<sub>2</sub>, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 5.6 mM glucose, and was adjusted to pH 7.4 with NaOH. The solution in the recording pipette was 80 mM KF, 60 mM K-acetate, 5 mM NaCl, 1 mM MgCl<sub>2</sub>, 10 mM ethylene glycol-bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), and 10 mM HEPES and was adjusted to pH 7.2 with KOH.

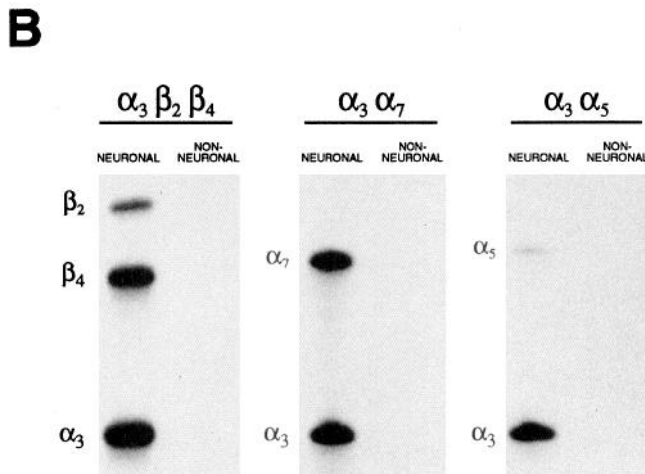
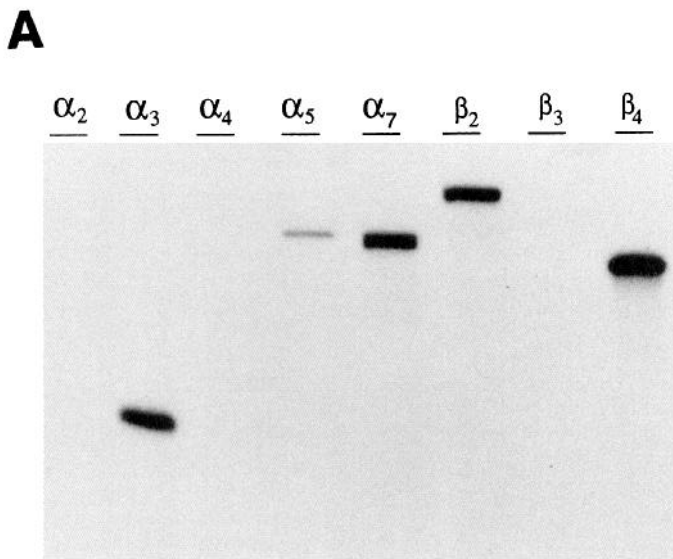
Standard *t* tests were done to assess the significance of differences between values obtained from denervated and control neurons, and between neurons of different ages.

## Results

To date, nine genes have been cloned that encode neuronal nAChRs subunits in mammals (Deneris et al., 1991; Role, 1992; Sargent, 1993): six  $\alpha$  subunits ( $\alpha 2$ – $\alpha 7$ ), whose designation is based on a pair of vicinal cysteines at positions equivalent to 192 and 193 of the muscle  $\alpha$  subunit, a location believed to be near the ACh binding site (Kao et al., 1984); and three  $\beta$  subunits ( $\beta 2$ – $\beta 4$ ), which lack these paired cysteines. Like muscle nAChRs, neuronal nAChRs are likely to be pentameric structures containing two  $\alpha$  subunits and three  $\beta$  subunits (Anand et al., 1991; Cooper et al., 1991).

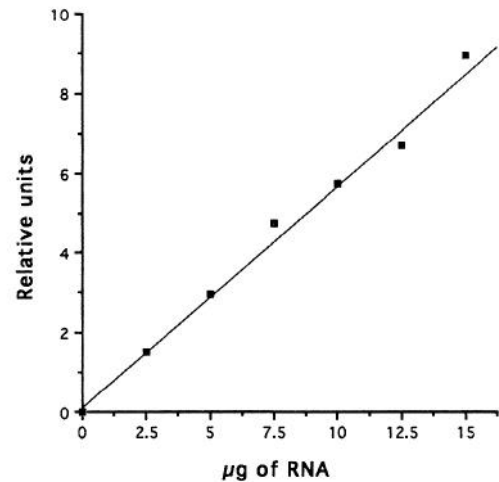
We used RNase protection assays to determine which neuronal nAChR transcripts are present in neonatal sympathetic neurons. As shown in Figure 1A, mRNA encoding five of the subunits are expressed in sympathetic neurons from P1 rats:  $\alpha 3$ ,  $\alpha 5$ ,  $\alpha 7$ ,  $\beta 2$ , and  $\beta 4$ . We quantified the hybridization signals with a phosphor imaging system, taking into account the specific activity of each riboprobe. These data indicate that the ratios of  $\alpha 3$  mRNA levels in P1 neurons relative to the other four subunits are approximately as follows:  $\alpha 5$ : $\alpha 3$ , 1:5;  $\alpha 7$ : $\alpha 3$ , 1:2;  $\beta 2$ : $\alpha 3$ , 1:2; and  $\beta 4$ : $\alpha 3$ , 3:2. We did not detect mRNA for  $\alpha 2$  or  $\alpha 4$ , nor did we detect mRNA for  $\beta 3$ . To ensure that  $\alpha 2$ ,  $\alpha 4$ , and  $\beta 3$  probes were capable of protecting the appropriate transcripts, we performed RNase protection assays on P1 rat brain RNA; our results indicate that all eight transcripts are present in neonatal brain (data not shown).

As we used whole ganglia in these experiments, it is conceivable that some transcripts might be expressed in the ganglionic non-neuronal cells. To demonstrate that the transcripts are spe-



**Figure 1.** nAChR subunit mRNAs expressed in rat SCG ganglia. *A*, Total cellular RNA was isolated from P1 rat SCG ganglia and 5  $\mu$ g was used in each RNase protection assay; a single radiolabeled probe was used for each reaction. *B*, P7 SCG were dissociated and the neurons and non-neuronal cells were separated by density centrifugation; 5  $\mu$ g of total cellular RNA, isolated from the two populations, was used in each RNase protection assay. This figure represents three different assays and shows that only the neuronal populations had detectable transcripts. The non-neuronal cell lanes also demonstrate that all probes were completely digested by RNase T1. The autoradiograms in *A* and *B* were exposed for 20 hr.

cifically localized to the neurons, we separated the neuronal and non-neuronal cell populations by density gradient centrifugation and assayed RNA isolated from both cell types separately. Over 90% of total cellular RNA from the ganglia was present in the neuronal fraction, a value that changes little over the first 2 postnatal weeks. Figure 1*B* shows that no transcripts were detected in RNA from P7 ganglionic non-neuronal cells, indicating that the nAChR subunit mRNAs in whole ganglia, such as shown in Figure 1*A*, are in the neurons. Similar results were also obtained with non-neuronal cells from P1 and P14 ganglia. In addition, preliminary *in situ* hybridization experiments indicate that individual neurons express all five nAChR transcripts (P. De Koninck and E. Cooper, unpublished observations).



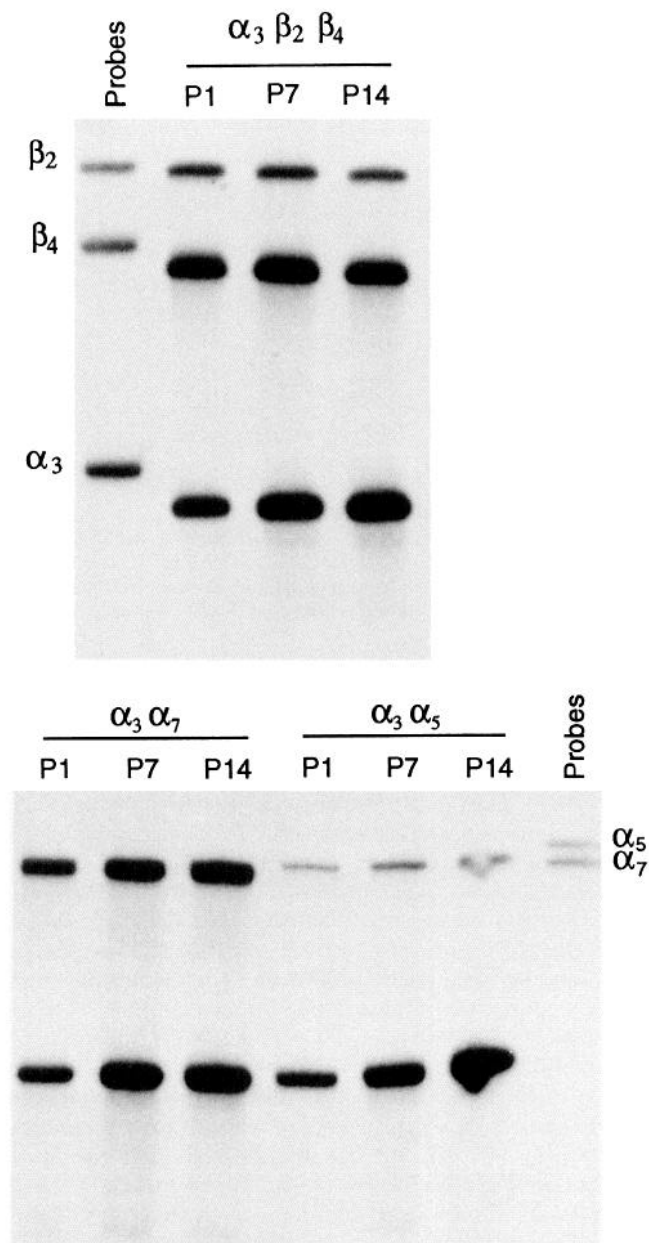
**Figure 2.** This figure shows the relative intensities of the hybridization signals from RNase protection assays for  $\alpha_7$  mRNA with increasing concentrations of RNA from SCG neurons. The hybridization signals were quantified with a phosphor imaging analyzer and are linear up to at least 15  $\mu$ g of total cellular RNA. Similar results were obtained for  $\alpha_3$ ,  $\alpha_5$ ,  $\beta_2$ , and  $\beta_4$ .

#### Increase nAChR subunit mRNAs with development

In the rat SCG, the number of synapses immediately after birth is less than 10% of that in the adult (Smolen and Raisman, 1980; Rubin, 1985). During the following 2–3 postnatal weeks, the number of synapses increase severalfold as the sympathetic neurons develop more extensive dendritic arbors (Smolen and Raisman, 1980; Schafer et al., 1983). Consequently, we asked whether nAChR mRNA levels reflect this developmental increase in cholinergic synapse formation. To quantify the changes in mRNA, we measured the hybridization signals with a phosphor imaging analyzer, and Figure 2 shows that the intensity of the hybridization signal increases linearly with increasing concentrations of RNA, thereby allowing us to make comparisons of signals over a similar range of intensities. Figure 3 shows an autoradiogram from a typical experiment in which we assayed SCG RNA from P1, P7, and P14 animals. Hybridization signals like those shown in Figure 3 were quantified from seven such experiments. Figure 4 shows the changes in mRNA levels over the first 2 postnatal weeks relative to the level at P1 for all five nAChR transcripts. The largest change occurs in  $\alpha_3$  mRNA: it increases 3.6-fold over the 2 week period.  $\alpha_7$  mRNA levels show the next largest change, increasing almost threefold, with most of the increase occurring during the first week. On the other hand, mRNA for  $\alpha_5$ ,  $\beta_2$ , and  $\beta_4$  transcripts showed little change over the 2 week period.  $\alpha_5$  mRNA levels appeared to increase 1.5-fold on average, but the increase was not statistically significant. As a result of the developmental changes in mRNA levels, at P14, the ratios of  $\alpha_3$  mRNA levels relative to the other four subunits are approximately as follows:  $\alpha_5$ : $\alpha_3$ , 1:12;  $\alpha_7$ : $\alpha_3$ , 2:5;  $\beta_2$ : $\alpha_3$ , 1:7; and  $\beta_4$ : $\alpha_3$ , 2:5. As is the case in P1 ganglia, mRNA for  $\alpha_2$ ,  $\alpha_4$ , or  $\beta_3$  was not detected in ganglia from P7 or P14 animals (data not shown).

#### Innervation differentially controls mRNA levels

It is well known that cutting motor nerves to skeletal muscle results in a large increase in extrajunctional nAChRs within a few days, a process referred to as “denervation supersensitivity” (Axelsson and Thesleff, 1959; Fambrough, 1979), and this in-



**Figure 3.** Postnatal development of nAChR transcripts. Total cellular RNA was isolated from P1, P7, and P14 SCG ganglia and 5  $\mu$ g was used in each RNase protection assay. The upper autoradiogram shows the protected transcripts for  $\alpha_3$ ,  $\beta_2$ , and  $\beta_4$  combined, and the lower autoradiograms show the protected transcripts for both  $\alpha_7$  and  $\alpha_3$ , as well as  $\alpha_5$  and  $\alpha_3$  combined. The probe lanes consist of 600 cpm of undigested probes. Both autoradiograms were exposed for 20 hr.

crease in functional nAChRs corresponds to a severalfold elevation in mRNA for the receptor subunits (Goldman et al., 1988; Brenner et al., 1990; Merlie et al., 1984). To determine whether similar changes occur in rat sympathetic neurons after denervation, we cut the preganglionic nerve to the SCG in P1 animals, and measured mRNA levels for nAChR transcripts from denervated sympathetic neurons 1 and 2 weeks later. Figure 5 shows data from one such experiment. The results from this and five other denervation experiments were measured relative to the contralateral controls as shown in Figure 6. We observed no significant difference between neurons denervated

for 1 week versus those denervated for 2 weeks, and consequently, Figure 6 includes data from both groups. Figure 6 indicates that the mRNA levels for all five transcripts in denervated neurons were less than that in control innervated neurons; however, the amount of change was different for the five. We observed the largest decrease in  $\alpha_7$  mRNA levels; it was only 64% of that in control neurons. These data indicate that much of the developmental increase for  $\alpha_7$  mRNA levels depends on preganglionic innervation. Similarly,  $\alpha_5$  mRNA levels were only 68% of controls. On the other hand,  $\alpha_3$  mRNA levels, which increase almost fourfold from P1 to P14, were 81% of control indicating that most of the developmental increase in  $\alpha_3$  mRNA levels is nerve independent.  $\beta$  subunit mRNA levels changed the least after denervation;  $\beta_4$  mRNA levels were 87% of control, and  $\beta_2$  mRNA levels were 89% of control. As in control innervated neurons, we failed to detect mRNA for  $\alpha_2$ ,  $\alpha_4$ , or  $\beta_3$  in denervated neurons (data not shown) demonstrating that the preganglionic nerves do not suppress the expression of these transcripts during postnatal development.

#### *Increases in ACh current densities correlate with increases in $\alpha$ -subunit mRNA levels*

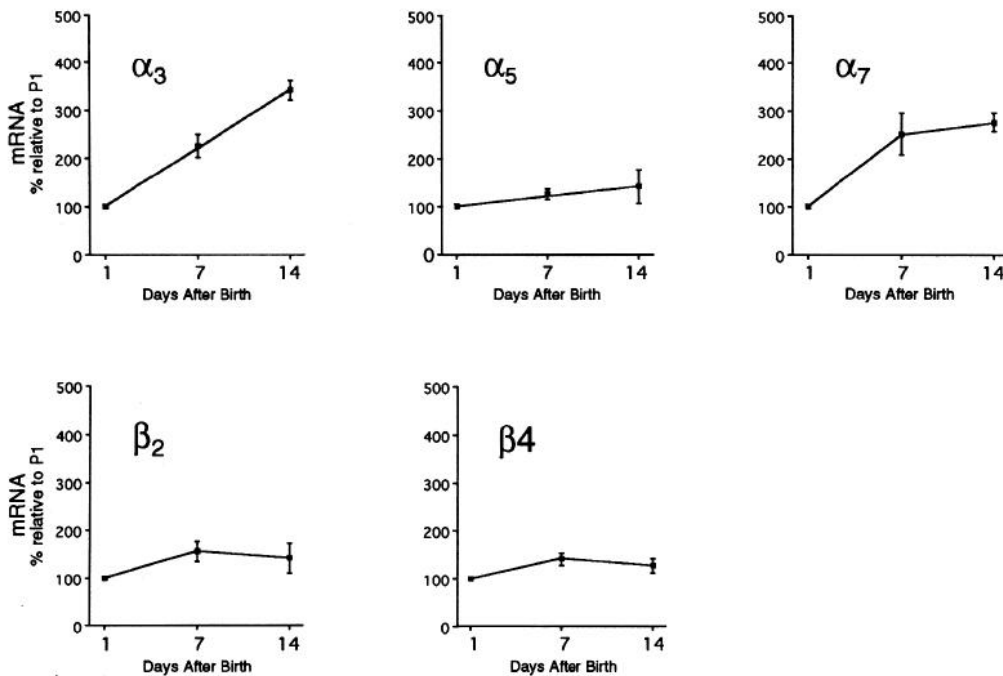
We used whole-cell voltage-clamp techniques to measure ACh current densities on SCG neurons during postnatal development and after denervation in order to correlate the appearance of functional nAChRs with the changes that we observed in mRNA levels for the different nAChR subunits. Figure 7 shows that the ACh current densities on neonatal rat SCG neurons increase nearly twofold from P1 to P14; this increase is a result of a fourfold increase in ACh current and a twofold increase in whole-cell capacitance. However, preganglionic innervation is not responsible for the postnatal increase in ACh current densities on these neurons; ACh current densities on neurons denervated for 2 weeks are not significantly different from those of control innervated neurons (see Fig. 7). These results indicate that the developmental increase in ACh current density is due to factors other than innervation.

#### **Discussion**

In this article, we demonstrate that mRNA levels for  $\alpha_3$  and  $\alpha_7$  subunits increase significantly over the first 2 postnatal weeks, and this increase correlates with an increase in ACh current densities. Our results also indicate that the preganglionic nerve differentially affects mRNA levels for the five nAChR transcripts expressed by neonatal rat sympathetic neurons, yet it has little influence on the ACh current densities.

We show that five nAChR transcripts are present in P1 rat sympathetic neurons,  $\alpha_3$ ,  $\alpha_5$ ,  $\alpha_7$ ,  $\beta_2$ , and  $\beta_4$ ; the mRNA levels for  $\alpha_3$  are approximately twofold greater than  $\alpha_7$  and  $\beta_2$ , fivefold greater than  $\alpha_5$ , but only 50% of that for  $\beta_4$ . These transcripts have also been shown to be expressed in chick autonomic neurons (Boyd et al., 1988; Couturier et al., 1990a; Listerud et al., 1991; Vernallis et al., 1993), and recently their amounts have been quantified in embryonic chick ciliary neurons (Corriveau and Berg, 1993). We did not detect mRNA for  $\alpha_2$ ,  $\alpha_4$ , or  $\beta_3$ , although mRNA for  $\alpha_4$  is present in embryonic chick sympathetic neurons (Listerud et al., 1991). A significant finding of our study is that during early postnatal development in the rat, there is a differential increase in mRNA levels for the various nAChR subunits present in sympathetic neurons. The greatest change occurs in  $\alpha_3$  mRNA levels, which increase nearly fourfold over the first 2 postnatal weeks. In addition, mRNA levels





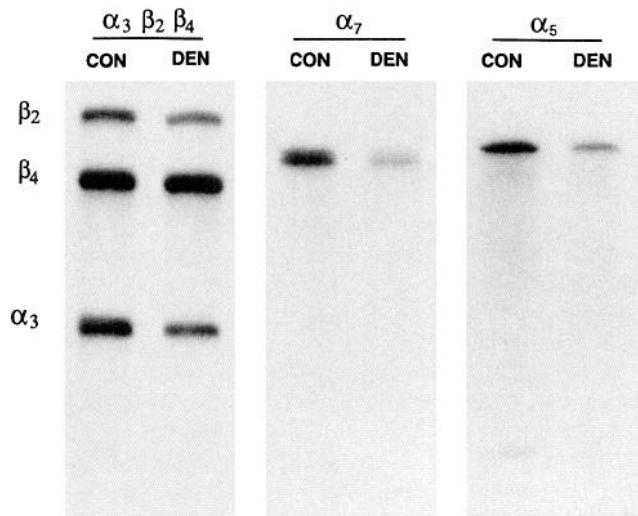
**Figure 4.** Developmental regulation of nAChRs subunit mRNA levels. This figure shows the change in mRNA, determined with RNase protection assays, for the five nAChR transcripts present in SCG neurons measured over the first 2 weeks of postnatal development. For each transcript, the values at P7 and P14 are relative to the values at P1. Each point represents the mean of 7–10 experiments, and the error bars represent the SEM.

for  $\alpha_7$  increase threefold over the same period. On the other hand, there is little or no change in mRNA levels for  $\alpha_5$ ,  $\beta_2$ , or  $\beta_4$ . As a result of these changes, at P14,  $\alpha_3$  mRNA is approximately 2.5-fold greater than  $\alpha_7$  and  $\beta_4$ , 7-fold greater than  $\beta_2$ , and almost 12-fold greater than  $\alpha_5$ .

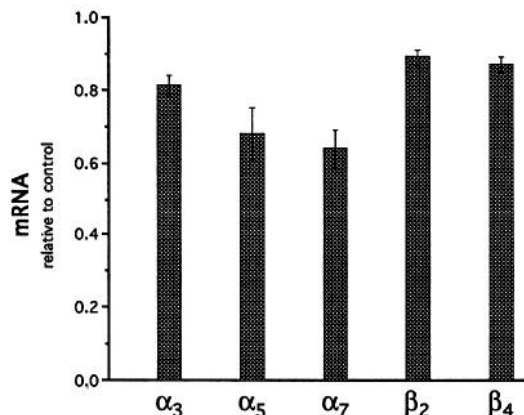
Our findings that during postnatal development mRNA levels for  $\alpha_3$ ,  $\alpha_5$ , and  $\beta_4$  increase differently are interesting because the three genes are clustered in the rat (Boulter et al., 1990) and chick (Couturier et al., 1990a) genome. In this cluster,  $\beta_4$  is

upstream from  $\alpha_3$ , and  $\alpha_5$  is further downstream and is transcribed in the opposite direction. Our results suggest that the regulation of mRNA levels encoding these three subunits is different, although we have not distinguished between regulatory mechanisms affecting transcription, RNA processing, or mRNA stability.

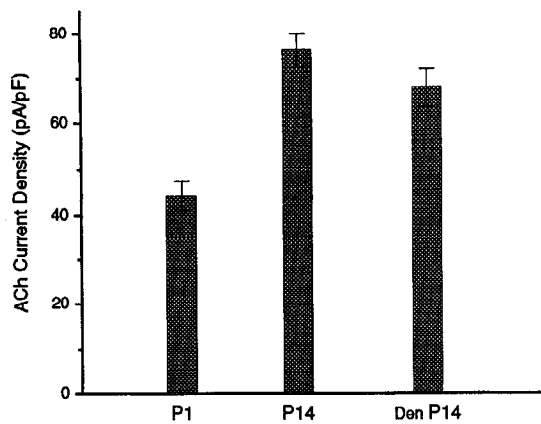
The changes in  $\alpha_3$  and  $\alpha_7$  mRNA levels correlate with changes in ACh current densities. We show that there is a twofold increase in ACh current densities over the first 2 postnatal weeks, a period when most synapses form on these neurons (Smolen and Raisman, 1980; Rubin, 1985). In addition, we show that denervation on P1 has little effect on the developmental increase in ACh current densities. We find that ACh current densities on denervated P14 neurons are not different from those on control neurons of the same age. The lack of effect of denervation



**Figure 5.** Differential regulation of nAChR transcripts following denervation. Total cellular RNA was isolated from both P14 innervated and P14 denervated SCG ganglia from the contralateral side and 5  $\mu$ g was used in each RNase protection assay. This figure shows three different autoradiograms; each autoradiogram compares protected nAChR transcripts from denervated SCG ganglia (DEN) with those from control innervated SCG ganglia (CON). The autoradiograms were exposed for 13 hr for the combination of  $\alpha_3$ ,  $\beta_2$ , and  $\beta_4$ ; for 20 hr for  $\alpha_7$ , and for 120 hr for  $\alpha_5$ .



**Figure 6.** Relative changes in mRNA levels for nAChR subunits after denervation. This figure shows the change in mRNA levels for all five nAChR transcripts after 1–2 weeks of denervation, as measured with RNase protection assays and quantified with a phosphor image analyzer. The values are relative to the levels in contralateral control neurons, and represent the mean of six experiments; the error bars are the SEM.



**Figure 7.** ACh current densities. This figure shows the mean ACh current densities for acutely dissociated P1 ( $n = 32$ ), P14 ( $n = 28$ ), and P14 neurons denervated for 2 weeks ( $n = 30$ ). ACh current densities increase during postnatal development independent of the presence of preganglionic innervation. Error bars represent SEM.

on current densities correlates with the small effect that denervation has on  $\alpha 3$ ,  $\beta 2$ , and  $\beta 4$  mRNA levels, but not for  $\alpha 5$  or  $\alpha 7$ , and, in fact, the absolute levels for all five transcripts are relatively abundant in denervated neurons. As these neurons had been already contacted by nerves before denervation, it is conceivable that the initial expression of nAChR genes in sympathetic neurons is induced by innervation, but thereafter, much of the expression is independent of innervation.

Our finding that the ACh current densities double during the period when most of the synapses are forming is consistent with previous studies on embryonic chick autonomic neurons (Maggiotta and Gurantz, 1989; Engisch and Fischbach, 1990). Furthermore, denervation of chick autonomic neurons does not affect ACh currents (McEachern et al., 1989; Engisch and Fischbach, 1992). In contrast to the 19% decrease in  $\alpha 3$  mRNA levels in rat sympathetic neurons,  $\alpha 3$  mRNA levels in chick ciliary neurons drop by 63% after denervation (Boyd et al., 1988); this correlates with a drop in the intracellular pool for nAChRs, but without altering the number of nAChRs at the surface (Jacob and Berg, 1988). The lack of effect of denervation on surface receptors is consistent with our results on ACh current densities.

Our results that  $\beta 2$  mRNA levels in rat sympathetic neurons change very little over the period of synapse formation and is largely unaffected by denervation at birth appear somewhat contrary to data on changes in  $\beta 2$  mRNA in the developing chick optic tectum. In the chick optic tectum, mRNA levels for  $\beta 2$  increase 10-fold during embryonic development, over the time when the retinal axons make synaptic contact in the tectum, and this increase in  $\beta 2$  mRNA is prevented if the eye is removed at embryonic day 2 (Matter et al., 1990). One possibility for this discrepancy is that  $\beta 2$  is regulated differently in different types of neurons. However, as mentioned above, in the rat SCG, the preganglionic axons had already innervated the sympathetic neurons at birth; conceivably, this initial contact could have induced the expression of  $\beta 2$ , and by birth,  $\beta 2$  expression loses its dependence on innervation.

Our results on mRNA encoding  $\alpha 7$  and  $\alpha 5$  need further discussion. We showed that the levels of  $\alpha 7$  mRNA increase threefold during postnatal development, and of the five transcripts present in rat sympathetic neurons, the  $\alpha 7$  mRNA levels are most affected by denervation at P1. It has been demonstrated

that  $\alpha 7$  mRNA encodes a subunit of the  $\alpha$ -bungarotoxin ( $\alpha$ -BTX) binding protein (Couturier et al., 1990b; Schoepfer et al., 1990; Seguela et al., 1993), yet the role for these proteins on neurons is not clear. Consistent with our results on  $\alpha 7$  mRNA, it has been shown that  $\alpha$ -BTX binding on chick autonomic neurons increases during development (Greene, 1976), and decreases after denervation (Jacob and Berg, 1987). However,  $\alpha$ -BTX does not block ACh-gated inward current on autonomic neurons (Brown and Fumagalli, 1977; reviewed by Chiappinelli, 1985), nor does it appear to be localized at the synaptic sites (Jacob and Berg, 1983; Loring et al., 1985), and in fact, recent evidence from chick ciliary neurons indicates that  $\alpha 7$  and  $\alpha 3$  are not part of the same receptor protein (Vernallis et al., 1993; but see Listerud et al., 1991). Furthermore, when  $\alpha 7$  mRNA is expressed in frog oocytes,  $\alpha 7$  proteins form a homomeric ligand-gated ion channel that is activated by ACh and nicotine and blocked by  $\alpha$ -BTX, and has a high permeability to calcium (Couturier et al., 1990b; Seguela et al., 1993). In addition, recent evidence suggests that when the  $\alpha$ -BTX binding proteins on chick ciliary neurons are activated by low agonist concentrations, as may occur during synapse formation, there is an increase in intracellular calcium concentrations (Vijayaraghavan et al., 1992). Perhaps during synapse formation the  $\alpha 7$ -containing proteins increase intracellular calcium, which subsequently regulates second messengers that modulate other proteins needed to establish the synapse (see Changeux, 1991).

The role for  $\alpha 5$  is less certain. There is evidence that  $\alpha 5$  codes for a sequence that binds  $\alpha$ -BTX (McLane et al., 1990), and therefore may be part of a  $\alpha$ -BTX binding protein on these neurons. To date, there is no evidence that mRNA for  $\alpha 5$ , either alone or in combination with other nAChR transcripts, can direct the synthesis of functional nAChRs when expressed in frog oocytes (Boulter et al., 1990; Couturier et al., 1990a). On the other hand, there is recent evidence that in chick ciliary neurons some nAChRs contain both  $\alpha 3$  and  $\alpha 5$  in the same receptor complex (Vernallis et al., 1993). We show that in sympathetic neurons from P1 rats,  $\alpha 5$  mRNA levels are approximately fivefold less than those for  $\alpha 3$ , and over the next 2 weeks, as the ACh current densities increase almost twofold, the ratio of  $\alpha 5$  to  $\alpha 3$  mRNA decreases almost threefold resulting in a  $\alpha 5$ : $\alpha 3$  ratio of roughly 1:12. It is likely that there are only two  $\alpha$  subunits in nAChRs (Anand et al., 1991; Cooper et al., 1991); if differences in mRNA levels reflect similar differences in protein levels, then our data on relative mRNA levels for  $\alpha 3$  and  $\alpha 5$  suggest that only a small proportion of the receptors would incorporate both  $\alpha 3$  and  $\alpha 5$ . However, further experiments are needed to establish if this is indeed the case.

It is interesting to compare our results on postnatal changes in mRNA levels for neuronal nAChR subunits in sympathetic neurons with changes that occur for nAChR in developing mammalian skeletal muscle. The initial expression of nAChR genes that occurs in muscle shortly after fusion is part of an endogenous myogenic program that activates all five muscle nAChR genes:  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  genes are activated at high levels, and the  $\epsilon$  gene at low levels (Piette et al., 1990; Martinou and Merlie, 1991; Jia et al., 1992; Prody and Merlie, 1992). Shortly after birth, a reciprocal change in  $\gamma$  and  $\epsilon$  mRNA levels occurs, such that by the second postnatal week,  $\epsilon$  mRNA increases roughly 10-fold whereas  $\gamma$ -mRNA levels drop to barely detectable levels (Mishina et al., 1986; Witzemann et al., 1989; Martinou and Merlie, 1991). Innervation does not appear to be responsible for the postnatal increase in  $\epsilon$  mRNA, but it is responsible for

the decrease in  $\gamma$  mRNA levels: for example, denervation at birth in the rat, and at P4 in the mouse, does not prevent the increase in  $\epsilon$  mRNA, but it does promote a large increase in  $\gamma$  mRNA; in addition, muscles denervated in adults of both species continue to express  $\epsilon$  mRNA (Witzemann et al., 1989; Brenner et al., 1990; Martinou and Merlie, 1991). This indicates that whereas the initial increase in  $\epsilon$  mRNA levels may depend on the motor nerve terminals; thereafter,  $\epsilon$  mRNA levels are independent of innervation. In rat sympathetic neurons, we did not observe a switch in the expression of the five neuronal nAChR subunit genes, and only mRNA for  $\alpha 3$  and  $\alpha 7$  showed a significant increase during the first 2 postnatal weeks. It would not be surprising if, like muscle, the initial expression of nAChR subunit genes is due to an intrinsic program that occurs in developing neuroblasts as they become committed to a particular neuronal phenotype. This would not necessarily exclude an influence from the ingrowing preganglionic terminals embryonically, but by birth, our results indicate that the expression of all five subunit genes is largely independent of innervation; this is similar to the  $\epsilon$  gene in muscle. Strict comparison between the regulation of nAChR subunit genes in sympathetic neurons and muscle is difficult, in part, because skeletal muscles are multinucleated cells, and the control of nAChR gene expression in the extrasynaptic nuclei is different from the control in the synaptic nuclei. For example, the  $\epsilon$  gene is selectively expressed by synaptic nuclei (Merlie and Sanes, 1985; Brenner et al., 1990), and in addition, the regulation of nAChR genes by the extrasynaptic nuclei, which express the  $\gamma$  gene, is controlled by innervation and activity (reviewed by Hall and Sanes, 1993). As such, it appears that the regulation of neuronal nAChR genes by rat sympathetic neurons during postnatal development resembles more closely the regulation of muscle nAChR genes by synaptic nuclei compared to regulation that occurs by extrasynaptic nuclei.

Lastly, we found no evidence for denervation supersensitivity, which has been shown to occur after denervation of many other targets, notably muscle. The lack of an increase in ACh current densities on denervated rat sympathetic neurons is consistent with recent findings on amphibian autonomic neurons (Dunn and Marshall, 1985; Streichert and Sargent, 1992). Similar to the lack of effect on ACh current density, denervation of sympathetic neurons at birth has little effect on either the growth of dendrites (Voyvodic, 1987), or on the developmental switch in voltage-gated K currents that occurs postnatally (McFarlane and Cooper, 1992). It may be that other factors regulate mRNA levels for nAChR subunits in these neurons, in particular, factors emanating from the targets. A likely target factor could be NGF, which has been shown to increase ACh current densities on rat nodose neurons in culture (Mandelzys and Cooper, 1992).

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