

Developing Neonatal Rat Sympathetic and Sensory Neurons Differ in Their Regulation of 5-HT₃ Receptor Expression

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Serotonin 5-HT₃ receptors (5-HT₃Rs) are ligand-gated ion channels expressed by many peripheral neurons and are involved in several physiological processes. To learn more about the developmental regulation of 5-HT₃R expression, we investigated rat sympathetic and vagal sensory neurons. We found that sympathetic and sensory neurons differ in their regulation of 5-HT₃R expression during early postnatal life and as these neurons develop in culture. In SCG neurons 5-HT₃R transcript levels are low at postnatal day 1 (P1) and increase 7.5-fold by P21; this increase occurs even after elimination of preganglionic innervation. In comparison, 5-HT₃R mRNA levels in P1 nodose neurons are over 14-fold greater than in P1 SCG and change little by P21. We show that 5-HT₃R transcript levels in nodose neurons depend on intact target innervation and drop by 60% after axotomy. When P1 SCG neurons develop in

culture, we observed a significant increase in 5-HT₃R expression: after 7 d in culture, transcript levels increase ninefold versus a threefold increase for neurons developing for 7 d *in vivo*. In contrast, 5-HT₃R mRNA levels in cultured nodose neurons drop by 70% within 24 hr; however, this drop is transient. After 2 d, transcript levels begin to increase, and after 7 d, they are above initial values. We show that this delayed increase in 5-HT₃R expression depends on neurotrophins. In both nodose and sympathetic neurons we found that the changes in 5-HT₃R gene expression correlate directly with the appearance of 5-HT-evoked current densities.

Key words: 5-HT₃ receptor; ligand-gated ion channel; sympathetic; superior cervical ganglion; sensory; nodose; trigeminal; mRNA expression; neurotrophins; axotomy

The serotonin 5-HT₃ receptor (5-HT₃R), a neurotransmitter-gated ion channel (Yakel and Jackson, 1988; Derkach et al., 1989; Maricq et al., 1991) present on many mammalian peripheral neurons, participates in several diverse physiological functions (Fozard, 1984; Jackson and Yakel, 1995). Activation of 5-HT₃Rs located on peripheral vagal sensory nerve endings initiates reflexes affecting respiration, circulation, emesis, and swallowing (Douglas, 1975; Sanders-Bush and Mayer, 1996). 5-HT₃Rs on spinal and vagal sensory neurons are involved in nociceptive signaling and nausea (Fozard, 1984). In the CNS 5-HT₃Rs are implicated in anxiety, depression, and drug dependence (Apud, 1993; Greenshaw, 1993). In addition, 5-HT₃Rs are expressed by sympathetic neurons; however, the role for these receptors in sympathetic function has not been fully determined (Wallis and North, 1978; Yang et al., 1992).

Many vagal afferent neurons expressing 5-HT₃Rs are located in the nodose ganglion. These sensory neurons have typical unipolar polarities; their axons bifurcate into a peripheral branch that innervates much of the viscera, including heart, lungs, trachea, and gut, and a central branch that terminates mainly in the nucleus tractus solitarius (Andresen and Kunze, 1994). The physiological responses to serotonin elicited from these vagal afferents depend, to a large extent, on the functional densities of 5-HT₃Rs

at these nerve endings. However, the factors and mechanisms that ensure the appropriate expression of 5-HT₃Rs on these nerve terminals are largely unknown.

Recent studies have shown that during embryonic development 5-HT₃R gene expression in nodose ganglia is observed as early as embryonic day 10 (E10) in rodents (Johnson and Heinemann, 1995; Tecott et al., 1995) and that by birth these ganglia contain abundant 5-HT₃R mRNA (Tecott et al., 1995). In contrast, 5-HT₃R expression in embryonic sympathetic neurons is low, and at birth few sympathetic neurons have detectable levels of 5-HT₃R mRNA (Johnson and Heinemann, 1995). These results suggest that embryonic sensory and sympathetic neurons use different mechanisms to regulate 5-HT₃R gene expression. Our interest is to learn more about factors and mechanisms that influence 5-HT₃R expression as peripheral neurons differentiate.

In this study we have asked (1) whether the expression of 5-HT₃Rs in vagal sensory neurons from the nodose ganglion changes during the first few postnatal weeks, a time when many autonomic reflexes become active; (2) whether 5-HT₃R expression depends on intact target innervation; (3) whether neurotrophins influence 5-HT₃R expression by nodose neurons; and (4) whether the regulation of 5-HT₃R expression in neonatal nodose neurons is similar to that which occurs in sympathetic neurons.

Our results demonstrate that 5-HT₃R transcript levels in neonatal nodose neurons are relatively abundant, change little over the first 3 postnatal weeks, and depend on target innervation. In comparison, we find that in SCG neurons 5-HT₃R transcript levels increase steadily and are 7.5-fold greater at P21 than at P1; this increase occurs even after elimination of the preganglionic innervation. Our results with SCG and nodose neurons in culture indicate that the regulation of 5-HT₃R expression by sympathetic

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neurons differs from that by nodose neurons during neonatal development.

An abstract of our initial results has appeared previously (Rosenberg et al., 1994).

MATERIALS AND METHODS

Tissue samples and RNA extraction

Superior cervical ganglia (SCG), nodose ganglia, and trigeminal ganglia were dissected from postnatal day 1 (P1), P7, P14, and P21 rats (Sprague Dawley CD strain, Charles River, Canada). Total RNA was prepared from these tissues, using the guanidium isothiocyanate-phenol-chloroform extraction method of Chomczynski and Sacchi (1987). The ganglia were placed into ice-cold L-15 media without sodium bicarbonate during the dissection, and then placed into GTC solution (+ β -mercaptoethanol) and homogenized for 10 sec with a Polytron (Brinkmann Instruments, Rexdale, Ontario, Canada). Total RNA was prepared from this lysate by standard phenol-chloroform extraction and ethanol precipitation.

SCG preganglionic denervation and nodose axotomy. The surgical procedures used to cut the preganglionic nerve to the SCG in P1 rats were similar to those described previously (Voyvodic, 1987; McFarlane and Cooper, 1992). Briefly, P1 rats were anesthetized by cooling on ice, and the right sympathetic trunk was exposed and crushed midway between the ganglion and the first rib. Then the trunk was cut rostral to the crush, and the caudal stump was pushed away ventrally to prevent regeneration. The animals were returned to their mother, and 2 weeks after the surgery the denervated SCG ganglia were dissected and total cellular RNA was prepared from the ganglia as described above. Total cellular RNA also was prepared from the contralateral control SCG ganglia, the preganglionic inputs of which were left intact. Similar surgical procedures were performed for nodose axotomy in P0 and P8 rats. A partial right nodose axotomy was performed by crushing and cutting the vagus nerve midway between the nodose ganglion and the first rib; the caudal stump was pushed away to prevent regeneration. After 24 hr the axotomized and control nodose ganglia were dissected, and total RNA was prepared as described above.

Neuronal cultures and RNA extraction

SCG and nodose ganglia were dissected from P1 animals and dissociated mechanically and enzymatically as previously described (Mandelzys and Cooper, 1992; McFarlane and Cooper, 1992). Briefly, the ganglia were dissected under sterile conditions from animals killed by cervical dislocation. The ganglia were incubated for 15 min at 37°C in HBSS (without Ca²⁺ or Mg²⁺) containing collagenase (1 mg/ml, type I, Sigma, St. Louis, MO) and dispase (2.4 mg/ml, grade II, Boehringer-Mannheim, Indianapolis, IN). Then the ganglia were transferred to a similar solution containing only dispase, and the ganglia were triturated gently every 15 min with a fire-polished Pasteur pipette for a total of 3–4 hr. After dissociation the cells were centrifuged through a 35% Percoll density gradient (Pharmacia, Dorval, Québec, Canada) to separate neurons from non-neuronal cells. The neuronal fraction was washed twice with L-15 medium supplemented with 10% horse serum and plated at a density of 5000–8000 cells/cm² onto laminin-coated (30 μ g/ml, overnight at 4°C; gift of Dr. S. Carbonetto, McGill University, Canada) Aclar coverslips (Allied Chemicals, Clifton, NJ) in modified Petri dishes. The Petri dishes (35 mm, Corning, Corning, NY) were modified by boring a 1.5 cm diameter hole in the bottom and then gluing Aclar coverslips from underneath with silicone rubber (3140 MIL-A-46146 RVT coating, Dow Corning, Midland, MI); this created a 2-mm-deep well with a volume of ~200 μ l. The neurons were grown in 1.5 ml of L-15 medium supplemented with 5% rat serum, vitamins, cofactors, penicillin, streptomycin, and sodium bicarbonate as previously described (Hawrot and Patterson, 1979). For SCG neurons the media was supplemented with nerve growth factor (2.5S NGF, 25 ng/ml). Nodose neurons were grown in the L-15 growth media described above either without added neurotrophins; with NGF, brain-derived neurotrophic factor (BDNF, 25 ng/ml; gift of Amgen, Thousand Oaks, CA), and neurotrophin-3 (NT-3, 25 ng/ml; gift of Amgen); or with NGF, BDNF, NT-3, and elevated potassium (final [K⁺] = 40 mM). After 2 d in culture, cytosine arabinofuranoside (Ara-C, 10 μ M; Sigma) was added to the cultures for 2–3 d to kill the few remaining non-neuronal cells. The cultures were maintained at 37°C in a humidified incubator with an atmosphere of 5% CO₂/95% air. The media were replaced every 3 d with fresh media. In some experiments we treated nodose neurons with 1 μ g/ml cycloheximide (1 in 5000 dilution of a 5

mg/ml ethanol solution; Sigma) for the first 10 or 24 hr after dissection; this concentration of cycloheximide previously has been shown to block protein synthesis in cultured neonatal SCG neurons (Martin et al., 1988). Cycloheximide was added to the plating media and all solutions used during the dissociation procedure. Sister cultures were treated with vehicle alone and showed no difference from untreated controls.

Total cellular RNA was prepared from cultures of P1 SCG and nodose neurons at day 0 (D0; immediately after dissociation), D1, D2, D4, D7, or D14 either with the RNeasy total RNA kit (Qiagen, Hilden, Germany) or with the guanidium isothiocyanate-phenol-chloroform extraction method as previously described (De Koninck and Cooper, 1995). Typically, four Petri dishes were pooled for each RNA sample. The yield of total RNA obtained with either RNA preparation method was similar and ranged from 2 to 3 μ g. Total RNA also was prepared from the non-neuronal cells in a similar way. Less than 10% of total cellular RNA from the ganglia was present in non-neuronal cell fractions; previously, we determined that this value changes little over the first 2 weeks of development for SCG (Mandelzys et al., 1994).

RNase protection assays

RNase protection assays were performed as described by Krieg and Melton (1987) with minor modifications (Mandelzys et al., 1994). Briefly, ³²P-UTP-labeled antisense RNA probes to rat 5-HT₃R and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were transcribed *in vitro* with T7 RNA polymerase from linearized plasmids containing portions of the cDNA clones. The 5-HT₃R antisense probe was synthesized from a partial rat 5-HT₃R cDNA clone (in pBluescript SK, Stratagene, La Jolla, CA; gift of Dr. P. Séguéla, Montreal Neurological Institute, Canada) obtained by RT-PCR amplification, using oligonucleotide primers for the mouse 5-HT₃R cDNA sequence (Maricq et al., 1991). This partial rat 5-HT₃R cDNA was sequenced and found to be identical to the published rat 5-HT₃R cDNA sequence (Miyake et al., 1995). The 5-HT₃R antisense probe protects 563 bases of the rat 5HT₃R transcript between positions 268 and 831 of the rat cDNA sequence (Miyake et al., 1995). Both nodose and SCG neurons express two alternatively spliced 5-HT₃R transcripts that encode receptor proteins that differ by five amino acids located between hydrophobic domains M3 and M4 at position 1153 (Miquel et al., 1995; Miyake et al., 1995); however, differences in the physiological role of the variants remain unclear (Downie et al., 1994). The probe we used detects both splice isoforms. The GAPDH antisense probe, synthesized from the mouse GAPDH (mouse pTRI-GAPDH, Ambion, Austin, TX), protects 316 bases of the rat GAPDH mRNA transcript. The thermal stabilities of the probes, based on their melting temperatures, were found to differ by <1°C.

The labeled riboprobes were gel-purified before use. For each reaction 1 μ g of total RNA was combined with radiolabeled 5-HT₃R probe (200,000 cpm) and GAPDH probe (30,000 cpm) and allowed to hybridize at 60°C overnight. The unhybridized single-stranded RNAs were digested with RNase T1 (Sigma), and the protected RNA/RNA hybrids were denatured and separated on 5% polyacrylamide 8 M urea gels. The gels were dried, exposed to a phosphorimaging screen (Fujix BAS 2000 and Molecular Dynamics, Sunnyvale, CA) to quantify the hybridization signals, and then exposed to x-ray film (Kodak XAR, Rochester, NY) with intensifying screens for 2–3 d at –80°C. The 5-HT₃R hybridization signals were normalized to those for GAPDH to take into account minor differences in the amount of RNA used per reaction. Each RNA sample was tested in duplicate or triplicate reactions. The relative amount of 5-HT₃R mRNA expression for each condition was the average of the values determined from three or more independently prepared RNA samples. The mRNA levels for both 5-HT₃R and GAPDH in P1 SCG and P1 nodose ganglia were similar to those of freshly dissociated D0 SCG and D0 nodose neurons, respectively, because the contribution of non-neuronal cells to the total ganglionic RNA was small (Mandelzys et al., 1994).

In situ hybridization

The *in situ* hybridization experiments were performed on tissue sections and on neuronal cell cultures according to the methods of Barthel and Raymond (1993) and Litman et al. (1993). Briefly, antisense digoxigenin (DIG)-labeled 5-HT₃R probe was synthesized by *in vitro* transcription with T7 RNA polymerase and DIG-11-UTP (Boehringer Mannheim) and the plasmid containing the portion of the rat 5-HT₃R cDNA described above. Because we were unable to quantify accurately the concentration of DIG-labeled probes with spectrophotometry, we measured

the amount of DIG-labeled antisense 5-HT₃R probe by RNase protection assays with ³²P-labeled sense 5-HT₃R probe; this also allowed us to verify that the *in vitro* transcription produced full-length DIG-labeled probes. To do this, we first synthesized unlabeled antisense 5-HT₃R cRNA, which we quantified by spectrophotometry, and then hybridized different amounts of unlabeled antisense 5-HT₃R cRNA with 200,000 cpm of ³²P-labeled sense 5-HT₃R to generate a standard curve. We then compared the hybridization signals obtained with the DIG-labeled antisense 5-HT₃R probe to the standard curve to determine the concentration of DIG-labeled antisense 5-HT₃R probe.

The *in situ* RNA hybridization experiments were performed on fresh-frozen cryostat sections of P5 and P21 SCG and nodose ganglia. The SCG and nodose ganglia were dissected together with the carotid artery, embedded in O.C.T. Compound (Tissue Tek, Miles, Elkhart, IN) and frozen in isopentane cooled on dry ice. The frozen tissue samples were cut at 16 μm thickness with a -20°C cryostat and placed onto Probe On Plus slides (Fisher Scientific, Pittsburgh, PA). Next, the sections were fixed for 5 min with 3% paraformaldehyde in 0.1 M Na phosphate buffer (0.1 M NaH₂PO₄/0.1 M Na₂HPO₄, pH 7.4), rinsed with PBS, rinsed with DEPC water, dehydrated with alcohol, air-dried, and stored at -80°C. The sections were warmed to room temperature and prehybridized for 3 hr at 43°C in a solution containing salmon sperm DNA (250 μg/ml, Pharmacia), yeast tRNA (500 μg/ml, Sigma), ribonucleoside vanadyl complex (20 mM, New England Biolabs, Beverly, MA), 4× SSC (0.3 M NaCl, 0.03 M Na citrate in DEPC water), 50% formamide, 3× Denhart's solution, 1% sarcosyl, and 20 mM Na phosphate buffer, pH 6.8. Then the prehybridization solution was replaced with the hybridization solution that contained the same ingredients plus dextran sulfate (10%), DTT (60 mM), and differing amounts of riboprobe (0.25–12.5 ng/μl). The sections were incubated at 43°C overnight, washed 3× for 15 min in 2× SSC at 43°C, and treated with RNase T1 (1000 U/ml) and RNase A (2 μg/ml; Pharmacia) in RNase buffer (0.3 M NaCl, 0.1 M Tris, pH 7.5, and 5 mM NaEDTA, pH 8.0) for 30 min at 37°C to digest unhybridized RNAs. The sections were rinsed with 1× SSC, 0.5× SSC, and 0.1× SSC for 15 min each at 43°C. The sections were rinsed with TBS, incubated for 1 hr at room temperature in TBS containing 1% blocking reagent for nucleic acids (Boehringer Mannheim), and then incubated for 1 hr with same buffer supplemented with 3.4 μl/ml of anti-DIG-alkaline phosphatase conjugate, Fab fragment (Boehringer Mannheim). The sections were rinsed 3× with TBS for 10 min each, and the bound probe was detected by standard alkaline phosphatase histochemistry. The reactions typically were developed for 18–24 hr in the dark. We performed controls with RNase T1 pretreatment or with DIG-labeled sense 5-HT₃R probe on tissue sections; these controls indicated that nonspecific signals were low. To verify the specificity of the 5-HT₃R probe, we pretreated the tissue sections with 400-fold excess unlabeled antisense 5-HT₃R probe, which competitively blocked the hybridization of the DIG-labeled antisense 5-HT₃R probe. Adjacent sections were stained with Masson's trichrome stain (Sigma) to identify the cellular components of the two ganglia.

In situ hybridizations also were performed on P1 SCG and nodose neurons grown in culture for 7 d. The nodose neurons were grown in the presence of NGF, BDNF, and NT-3 as described above. The cell cultures were fixed for 10 min with 4% paraformaldehyde in 0.1 M Na phosphate buffer, pH 7.4, rinsed with PBS, and stored in 70% ethanol at -20°C. The cultured neurons were permeabilized with ice-cold 100% ethanol, rehydrated with PBS, and then prehybridized and hybridized as described above. We performed similar controls as described above with 400-fold excess unlabeled 5-HT₃R antisense probe, with RNase T1 pretreatment, or with DIG-labeled sense 5-HT₃R probe.

Electrophysiology recordings and data analysis

5-HT-evoked current densities were measured with whole-cell patch-clamp techniques (Hamil et al., 1981). The currents were recorded at room temperature (22–24°C) with a List EPC-7 amplifier, filtered at 1.5 kHz with an eight-pole Bessel filter (Frequency Devices, Haverhill, MA), and then sampled at 5 kHz, displayed, and stored on-line with a 386-based PC computer (AT class running at 33 MHz and a 64K cache and A/D card; Omega, Stamford, CT). The program PATCHKIT (Alembic Software, Montréal, Canada) was used for data acquisition and analysis. Pipette resistances ranged from 2 to 6 MΩ and were balanced to zero with the pipette immersed in the perfusion solution. Neurons were voltage-clamped at -60 mV, and 5-HT (100 μM or 1 mM) was applied with a rapid agonist application method as previously described (Mandelzys et al., 1995). Tetrodotoxin (TTX) was added to all solutions to eliminate unclamped Na⁺ currents. In some nodose neurons 5-HT

application evoked a burst of TTX-insensitive Na⁺ currents that were superimposed on 5-HT-evoked currents; however, because of the rapid time course of the Na⁺ currents, they did not interfere with our ability to measure the amplitude of the 5-HT-evoked currents. The 5-HT-evoked current densities were calculated by dividing the peak current by the whole-cell capacitance; the whole-cell capacitance was obtained by integrating the capacity current evoked by a 5 mV hyperpolarizing pulse from a holding potential of -60 mV. The control perfusion solution consisted of (in mM) 140 NaCl, 5.4 KCl, 0.33 NaH₂PO₄, 0.44 KH₂PO₄, 0.28 CaCl₂, 0.18 MgCl₂, 10 HEPES, 5.6 glucose, and 2 glutamine with 5 μg/ml phenol red and 0.5 μM TTX (Sigma); pH was adjusted to 7.4. For drug application we used an identical solution in which we added 5-HT (0.1–1 mM). The pipette solution contained (in mM) 65 KF, 55 KAc, 5 NaCl, 0.2 CaCl₂, 1 MgCl₂, 10 EGTA, and 10 HEPES; pH was adjusted to 7.4. During the recording the cultures were perfused at 1 ml/min with control perfusion solution.

Statistical analysis. The results are expressed as the mean ± SEM, and Student's *t* tests were used to assess statistical significance.

RESULTS

We present our results on 5-HT₃R gene expression in neonatal SCG and nodose neurons in two parts: (1) during early postnatal development *in vivo* and (2) as these neurons develop in culture.

5-HT₃R gene expression in neonatal SCG and nodose neurons *in vivo*

5-HT₃R mRNA levels increase in SCG during early postnatal development

We examined 5-HT₃R gene expression in rat SCG during the first 3 weeks of postnatal development, using RNase protection assays (Fig. 1A). For each reaction we assayed GAPDH mRNA levels as internal controls and quantified the hybridization signals with a phosphorimaging system. Figure 1B shows the changes in 5-HT₃R mRNA levels relative to those at P1. These results demonstrate that 5-HT₃R gene expression increases steadily over the first 3 postnatal weeks such that by P21 the levels are 7.5-fold greater than at P1.

To verify that 5-HT₃R transcripts are present only in neurons of the SCG, we dissociated P1 SCG, separated neurons from non-neuronal cells by using a density gradient, and measured 5-HT₃R mRNA levels in both cell fractions. Our results indicate that these transcripts are detectable only in the neuronal fractions (data not shown).

Influence of preganglionic innervation. During the first 3 postnatal weeks preganglionic nerve terminals form 90% of their adult number of synapses on SCG neurons (Smolen and Raisman, 1980). To determine whether the increase in 5-HT₃R gene expression in SCG neurons during postnatal development is a consequence of increasing innervation, we cut the preganglionic nerve at P1 and measured 5-HT₃R mRNA levels in the denervated neurons 2 weeks later. We found that the 5-HT₃R mRNA levels in denervated neurons were not significantly different (*p* > 0.2) from innervated contralateral or unoperated P14 SCG neurons (Fig. 1C). These results indicate that the developmental increase in 5-HT₃R gene expression in SCG neurons during the postnatal period does not require preganglionic innervation.

5-HT₃R transcripts are differentially expressed in SCG and nodose ganglia

To determine what proportion of neurons in the ganglia express 5-HT₃R transcripts, we performed *in situ* hybridization experiments, using DIG-labeled riboprobes. Because one of the objectives of our study was to compare 5-HT₃R expression in sympathetic neurons with that in sensory neurons, we used tissue sections that included both the SCG and nodose ganglion (Fig. 2). Our results from P5 and P21 ganglia indicate that most SCG

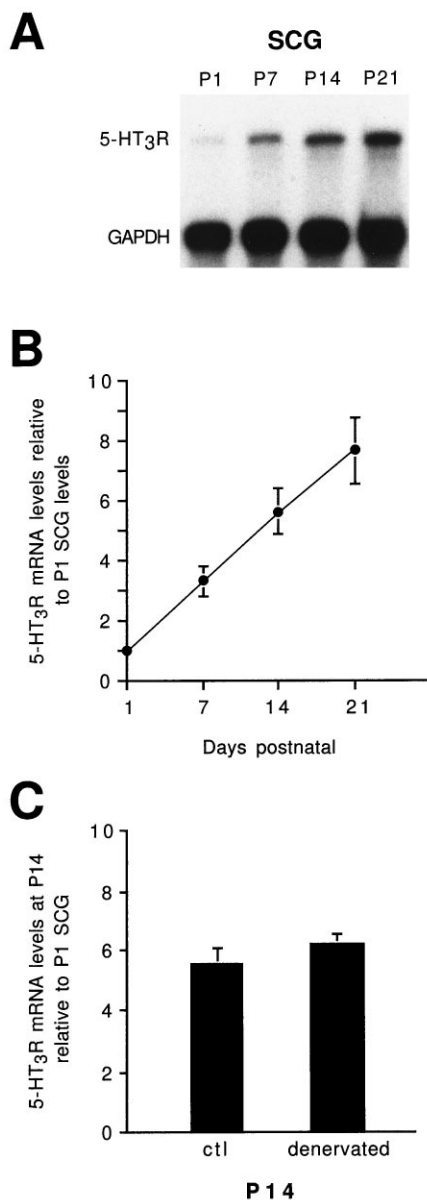


Figure 1. Developmental increase in 5-HT₃R mRNA expression in SCG. *A*, RNase protection assay for 5-HT₃R and GAPDH mRNA expression in total RNA isolated from P1, P7, P14, and P21 rat SCG. Total RNA (1 μg) was used for each reaction; the protected riboprobe sizes are 563 bases for 5-HT₃R and 316 bases for GAPDH. GAPDH mRNA expression was assessed to ensure that equal amounts of total RNA were used in each reaction. *B*, Quantification of 5-HT₃R mRNA expression in SCG: 5-HT₃R expression increases 7.5-fold during the first 3 weeks of postnatal life. *C*, This figure shows that when P1 SCG ganglia are denervated for 2 weeks, there is no significant change in 5-HT₃R mRNA expression, suggesting that innervation does not contribute to the observed developmental increase in 5-HT₃R expression. The values represent the mean of six to eight experiments, and the error bars represent the SEM.

neurons express 5-HT₃R mRNA and that the levels in these neurons increase with development, as we show quantitatively with RNase protection assays (see Fig. 1). Figure 2 also shows that nodose neurons contain abundant 5-HT₃R transcripts, consistent with previous studies reporting 5-HT₃R mRNA expression in embryonic nodose neurons (Johnson and Heinemann, 1995; Tecott et al., 1995). The medial neurons in nodose ganglia appear to have lower amounts of 5-HT₃R mRNA than the lateral neu-

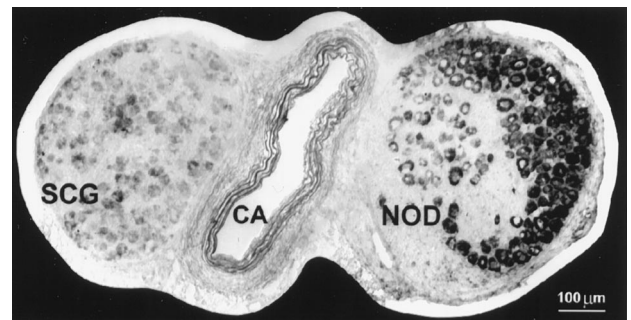


Figure 2. Differential 5-HT₃R mRNA expression in SCG and nodose neurons. *In situ* hybridization with DIG-labeled antisense 5-HT₃R probe on a section through P21 rat superior cervical ganglia (SCG), carotid artery (CA), nodose (NOD), and associated connective tissue. This figure is a montage of photomicrographs taken with a 20× objective and DIC optics on a Zeiss Axiovert 35 microscope. This figure shows that 5-HT₃R mRNA is expressed at high levels in most nodose neurons and at lower levels in most SCG neurons and is not detectable in non-neuronal cells.

rons. This pattern may result from different target innervation. In cat and rabbit, nodose neurons that project to specific targets are organized topographically within the ganglion (Mei, 1970; Portatier and Vigier, 1979; Donoghue et al., 1982). However, comparable experiments have not been done in rat. Our results with *in situ* hybridizations also confirm that 5-HT₃R transcripts are present only in the neurons and not in the non-neuronal cells of either ganglia.

5-HT₃R mRNA levels in nodose neurons change little during early postnatal development

We quantified 5-HT₃R mRNA levels in nodose neurons during postnatal development, using RNase protection assays (Fig. 3*A,B*). In contrast to the developmental increase observed in SCG, 5-HT₃R mRNA levels in nodose neurons changed little over the first 3 postnatal weeks. To compare the expression between nodose and SCG, we normalized 5-HT₃R mRNA hybridization signals to those of GAPDH. At P1 we find that 5-HT₃R mRNA levels in nodose are over 14-fold greater than in SCG (Fig. 3*C,D*); however, at P21 the 5-HT₃R mRNA levels in nodose are only threefold higher (Fig. 3*D*) because, as we have shown above, the levels in SCG increase significantly during the first few weeks.

Our results indicate that regulation of 5-HT₃R gene expression in nodose neurons differs from that in SCG neurons. To determine whether other sensory neurons regulate 5-HT₃R expression in a similar manner to nodose neurons, we measured 5-HT₃R mRNA levels in trigeminal ganglia. Our results indicate that, unlike nodose neurons, 5-HT₃R mRNA levels in trigeminal ganglia are low in P1 animals, increase steadily over the first 3 postnatal weeks (data not shown), and are four- to fivefold greater at P21 (Fig. 3*D*). These results suggest that 5-HT₃R gene expression is not identical in all sensory neurons.

Effects of axotomy. One factor known to regulate gene expression in peripheral sensory neurons is target innervation (Herdgen and Zimmerman, 1994; Hökfelt et al., 1994*a,b*). To determine whether target innervation influences 5-HT₃R transcripts levels in nodose neurons, we cut the vagus nerve distal to the ganglion and then quantified 5-HT₃R mRNA levels in the axotomized neurons 24 hr later (Fig. 4). Cutting the vagus nerve in P0 animals resulted in a 60% decrease in 5-HT₃R mRNA levels

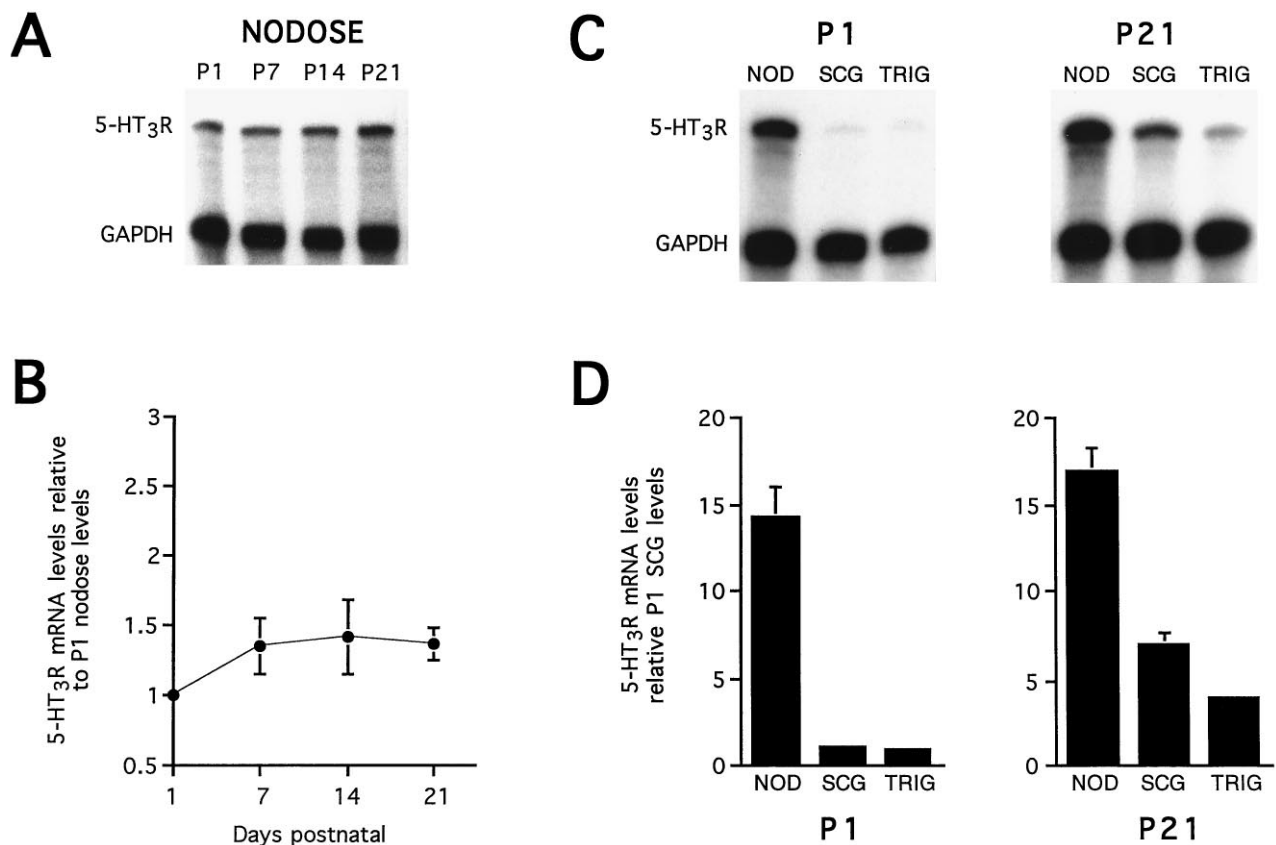


Figure 3. 5-HT₃R mRNA is highly expressed during early postnatal development in nodose ganglia. *A*, RNase protection assay for 5-HT₃R mRNA expression performed as in Figure 1 on 1 μ g of total RNA isolated from P1, P7, P14, and P21 rat nodose ganglia. *B*, Quantification of 5-HT₃R mRNA expression in nodose: 5-HT₃R expression changes little during the first 3 weeks of postnatal development. *C*, RNase protection assay depicting a comparison of 5-HT₃R mRNA expression in 1 μ g of total RNA prepared from P1 and P21 nodose, SCG, and trigeminal ganglia. *D*, Quantification of 5-HT₃R mRNA expression in nodose (NOD), SCG, and trigeminal ganglia (TRIG) at P1 and P21. The values represent the mean of six to eight experiments, and the error bars represent the SEM. In P1 SCG, P1 NOD, and P21 TRIG, the error bars are too small to resolve. This figure shows that at P1 5-HT₃R expression is 14-fold higher in nodose than in SCG or trigeminal. However, in SCG and trigeminal, 5-HT₃R mRNA expression increases significantly during the first 3 postnatal weeks such that by P21 the levels in nodose are only threefold larger than in SCG and fourfold larger than in trigeminal.

relative to contralateral control neurons. This experiment was done also on P8 animals, and as we found a similar decrease after axotomy, we combined the results from both groups in Figure 4*B*. These findings indicate that 5-HT₃R mRNA expression in postnatal nodose neurons depends on intact target innervation.

5-HT₃R gene expression in neonatal SCG and nodose neurons in culture

Most nodose and SCG neurons developing in culture express 5-HT₃R mRNA

To learn more about the regulation of 5-HT₃R gene expression in peripheral neurons, we investigated sympathetic and nodose neurons developing in culture. Figure 5 shows examples of *in situ* hybridization experiments with DIG-labeled 5-HT₃R probes on P1 nodose and SCG neurons that had developed in culture for 1 week in the virtual absence of ganglionic non-neuronal cells. Figure 5 shows that most SCG and nodose neurons in these cultures express 5-HT₃R transcripts. To demonstrate that the hybridization signals represent specific 5-HT₃R transcripts, we preincubated nodose cultures with 400-fold excess unlabeled antisense 5-HT₃R cRNA, which competitively blocked hybridization of the DIG-labeled antisense 5-HT₃R riboprobe (Fig. 5*C*). In addition, pretreatment of the cultures with RNases prevented

antisense riboprobe hybridization; similarly, DIG-labeled sense riboprobes did not hybridize (data not shown).

5-HT₃R gene expression increases in neonatal SCG neurons developing in culture

Figure 6 shows that 5-HT₃R mRNA levels increase in P1 SCG neurons developing in culture. The specific increase in 5-HT₃R mRNA levels in cultured neurons is greater than that *in vivo*: after 7 d in culture, transcript levels have increased ninefold, whereas after a similar time *in vivo* there is only a threefold increase (see Fig. 1*B*). This difference may reflect either that a factor(s) in these cultures stimulates 5-HT₃R gene expression or that an extrinsic factor(s) *in vivo* modulates its expression.

5-HT₃R gene expression decreases rapidly and transiently in cultured nodose neurons

Because neonatal rat nodose neurons express mRNA for *trkA*, *trkB*, *trkC*, and p75 receptors (M. R. and E. C., unpublished observations), we grew P1 nodose neurons in culture under conditions similar to those of SCG neurons, except that we supplemented the growth media with BDNF and NT-3 in addition to NGF (all at 25 ng/ml). Within 24 hr after plating, 5-HT₃R mRNA levels in cultured nodose neurons drop to 30% of initial values

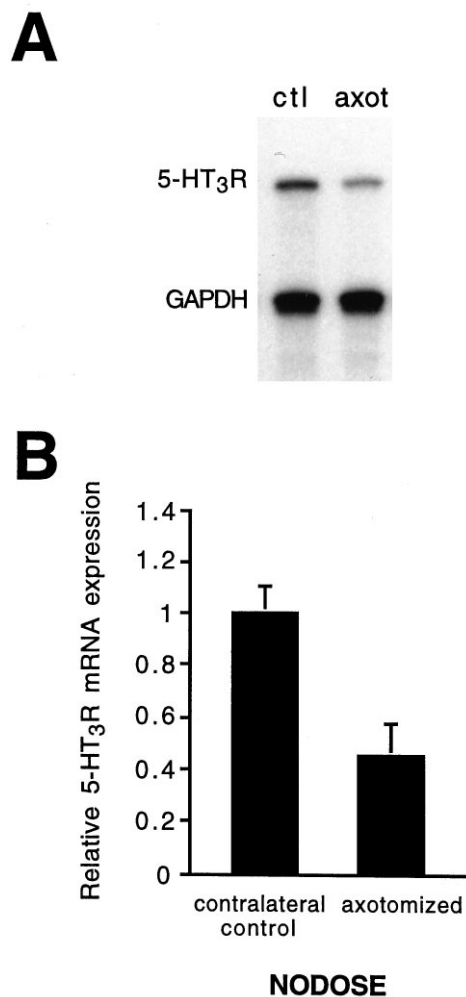


Figure 4. 5-HT₃R gene expression in nodose neurons depends on intact target innervation. *A*, RNase protection assay performed as in Figure 1 on 1 μ g of total RNA prepared from P1 nodose ganglia that were axotomized 24 hr earlier (*axot*) and from control contralateral P1 nodose ganglia (*ctl*). *B*, Mean \pm SEM 5-HT₃R mRNA levels for axotomized P1 and P9 nodose ganglia are significantly different from control contralateral ganglia ($p < 0.001$; $n = 12$).

(Fig. 7*A*). In early experiments we supplemented the media with only NGF or BDNF and observed similar decreases (data not shown). This rapid decrease in 5-HT₃R mRNA levels is similar to that observed in axotomized neurons *in vivo* and presumably occurs because the axons of these neurons are cut when the neurons are placed in culture.

Lack of effect of elevated K⁺ on the initial decrease. In a previous study we observed a significant decrease in mRNA levels for the α_7 neuronal nicotinic acetylcholine receptor (nAChR) subunit in cultured SCG neurons (De Koninck and Cooper, 1995), similar to the decrease we observed in 5-HT₃R mRNA levels in cultured nodose neurons. We showed that the decrease in α_7 mRNA in SCG neurons could be prevented by culturing the neurons in elevated (40 mM) K⁺; the resultant depolarization increased intracellular Ca²⁺ levels and increased α_7 mRNA levels by activating a Ca²⁺/calmodulin-dependent kinase (CaM kinase) pathway (De Koninck and Cooper, 1995). To test whether similar mechanisms regulate 5-HT₃R gene expression, we cultured nodose neurons in elevated K⁺; however, this did not prevent the

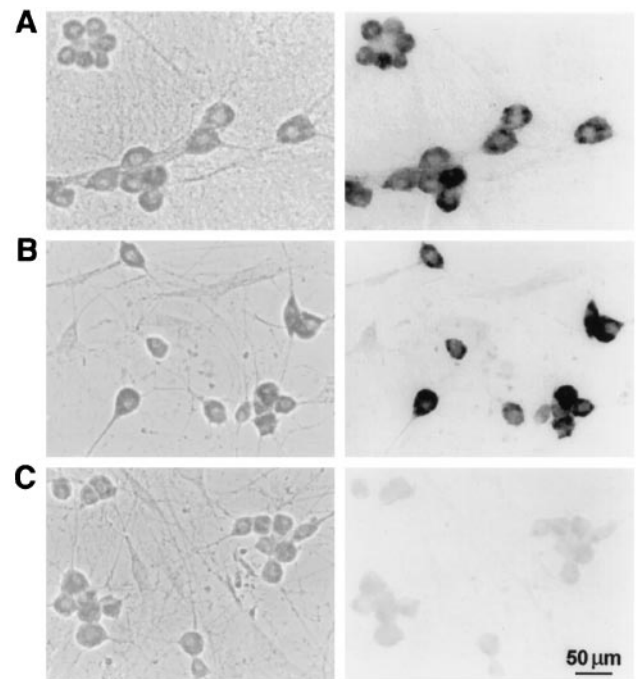


Figure 5. Most SCG and nodose neurons in culture express 5-HT₃R transcripts. *In situ* hybridization with DIG-labeled riboprobe for 5-HT₃R mRNA expression in P1 SCG and nodose neurons grown in culture for 7 d: *A*, SCG; *B*, nodose; *C*, control nodose neurons prehybridized with 400-fold excess unlabeled antisense 5-HT₃R probe. Phase contrast (*left*) and DIC (*right*) photomicrographs were taken with a 40 \times objective on a Zeiss Axiovert 35 microscope. 5-HT₃R mRNA is expressed by most D7 SCG and D7 nodose neurons.

rapid drop in 5-HT₃R mRNA levels (Fig. 7*B*), indicating that increases in intracellular Ca²⁺ are unlikely to regulate 5-HT₃R mRNA levels in cultured nodose neurons.

The decrease 5-HT₃R mRNA levels requires protein synthesis. We added cycloheximide, a protein synthesis inhibitor, to cultures of P1 nodose neurons for the first 24 hr after plating; this treatment mainly prevented the drop in 5-HT₃R mRNA levels, indicating that this decrease in nodose neurons is an active process that depends on *de novo* protein synthesis (Fig. 7*B*). In addition, treating neurons with cycloheximide for only the first 10 hr partially prevented the drop in 5-HT₃R mRNA levels: the levels were 45% of initial levels after 24 hr and 58% of initial levels after 48 hr in culture (data not shown).

Interestingly, SCG neurons grown in culture show no such decrease in mRNA levels; this observation further demonstrates that 5-HT₃R gene expression is differentially regulated in these two types of neurons.

Neurotrophins increase 5-HT₃R gene expression in cultured nodose neurons

We observed that the decrease in 5-HT₃R mRNA levels in cultured nodose neurons is transient: after 2 d in culture, transcript levels begin to increase, and after 7 d in culture, 5-HT₃R mRNA levels are above their initial levels (Fig. 7*A*). Because the neurotrophins NGF, BDNF, and NT-3 were present in these cultures, we asked whether these neurotrophins stimulated the delayed increase in 5-HT₃R gene expression. Because nodose neurons do not require exogenously supplied neurotrophins to grow in culture (Mandelzys and Cooper, 1992; De Koninck et al., 1993), we grew P1 nodose

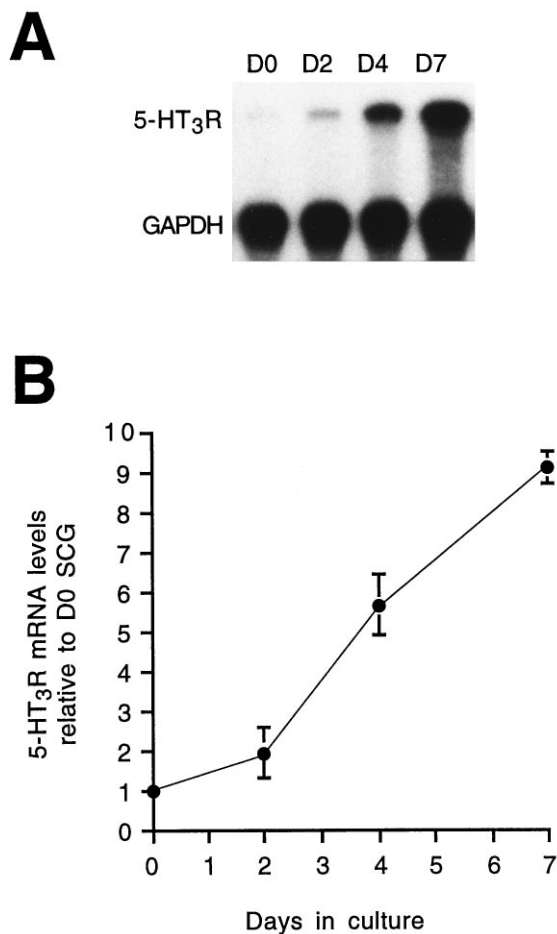


Figure 6. Developmental expression of 5-HT₃R transcripts by neonatal SCG neurons in culture. *A*, RNase protection assay performed as in Figure 1 on 1 μ g of total RNA extracted from freshly dissociated SCG day 0 (D0) neurons and SCG neurons grown in culture for 2, 4, and 7 d. *B*, Quantification of 5-HT₃R mRNA in cultured SCG neurons relative to D0. The values represent the mean of six to eight experiments, and the error bars represent the SEM.

neurons for 2 d without neurotrophins, allowing the drop in 5-HT₃R mRNA to occur, and then we divided the cultures into three groups. We used one group to measure 5-HT₃R mRNA levels at D2. We added 25 ng/ml of NGF, BDNF, and NT-3 to the second group and allowed the neurons to grow for an additional 5 d. We grew the third group for an additional 5 d in the absence of neurotrophins. The number of neurons in all three groups did not differ by >10%. In the group receiving neurotrophins, the 5-HT₃R mRNA levels at D7 were 4.5-fold greater than those at D2 (Fig. 7C), whereas in the group that did not receive neurotrophins, the levels were only 1.3-fold greater than those at D2. These results suggest that neurotrophins stimulate 5-HT₃R expression in these neurons.

Correlation of 5-HT₃R mRNA levels with 5-HT-evoked current densities

To determine whether changes in 5-HT₃R gene expression affect the appearance of functional 5-HT₃Rs on these neurons, we measured 5-HT-evoked currents on P1 nodose and SCG neurons developing in culture (Fig. 8A). We examined nodose neurons at two different times: early, after 1–3 d in culture, when 5-HT₃R mRNA levels in these neurons are low; and after 6–14 d in culture,

when 5-HT₃R mRNA levels are four- to fivefold larger. Figure 8B shows the mean 5-HT-evoked current densities for nodose neurons in culture for 1–3 d ($n = 36$) and for 6–14 d ($n = 25$) indicating that 5-HT-evoked current densities increase with time in culture. After 1–3 d in culture 83% of neurons (30 of 36) had 5-HT-evoked current densities below detectable levels (<1 pA/pF), whereas the mean 5-HT-evoked current density of the remaining 17% was 12.7 pA/pF \pm 2.6 SEM. In comparison, after 6–14 d in culture only 20% of neurons (5 of 25) had current densities <1 pA/pF; the mean 5-HT-evoked current density of the remaining 80% was 36.5 pA/pF \pm 6.9 SEM. These results demonstrate that changes in 5-HT₃R gene expression correlate directly with the appearance of functional 5-HT₃Rs on nodose neurons. We found a similar correlation for SCG neurons: most SCG neurons in culture for 1–3 d had small 5-HT-evoked current densities, and these 5-HT-evoked current densities increased over the first week in culture.

DISCUSSION

In this study we have investigated factors and mechanisms that influence 5-HT₃R expression as sympathetic and nodose neurons differentiate during early postnatal life, a time when many autonomic reflexes become active. By comparing sympathetic and nodose neurons, we have identified cell type-specific mechanisms that regulate 5-HT₃R gene expression during neonatal development and as the neurons differentiate in culture. These mechanisms are likely to play a role in determining the appropriate spatial and temporal expression of 5-HT₃Rs in sensory and sympathetic neurons during early postnatal life.

Our results demonstrate that neonatal nodose and SCG neurons differ in 5-HT₃R mRNA expression. This differential expression may be attributable to the embryological origins of these two neuronal populations: SCG neurons originate from the neural crest, whereas nodose neurons are derived from the placodes (Le Douarin, 1984). Nodose neurons *in vivo* express high levels of 5-HT₃R mRNA: at P1, the levels are 14-fold greater than in SCG neurons and change little over the first 3 postnatal weeks. Presumably, these high levels ensure that nodose neurons express appropriate densities of functional 5-HT₃Rs at birth. The 5-HT₃R mRNA levels in sympathetic neurons increase 7.5-fold during the first 3 weeks of postnatal development. This increase may be related to increasing sympathetic target innervation during this neonatal period (Black, 1978). Less is known about the postnatal development of target innervation by rat nodose neurons. The lack of change in 5-HT₃R mRNA levels in nodose neurons during the first 3 postnatal weeks suggests either (1) that similar factors regulate 5-HT₃R expression in SCG and nodose neurons and that target innervation by vagal afferent neurons is essentially complete by birth or (2) that both types of neurons are increasing their target innervation during neonatal development, yet the factors that regulate 5-HT₃R gene expression in nodose and SCG neurons are different.

The increase in 5-HT₃R mRNA levels in SCG neurons *in vivo* is much greater than the increase in neuronal nAChR subunit transcripts expressed by these neurons. We showed previously that the expression of the α_3 and α_7 nAChR subunits increases four- and threefold, respectively, over a similar postnatal period, whereas as the expression of the other nAChR subunits, β_2 , β_4 , and α_5 , does not change significantly (Mandelzys et al., 1994). This indicates that in SCG neurons the regulation of 5-HT₃R gene expression differs from that of nAChR subunits. In addition, we show that the developmental increase in 5-HT₃R mRNA

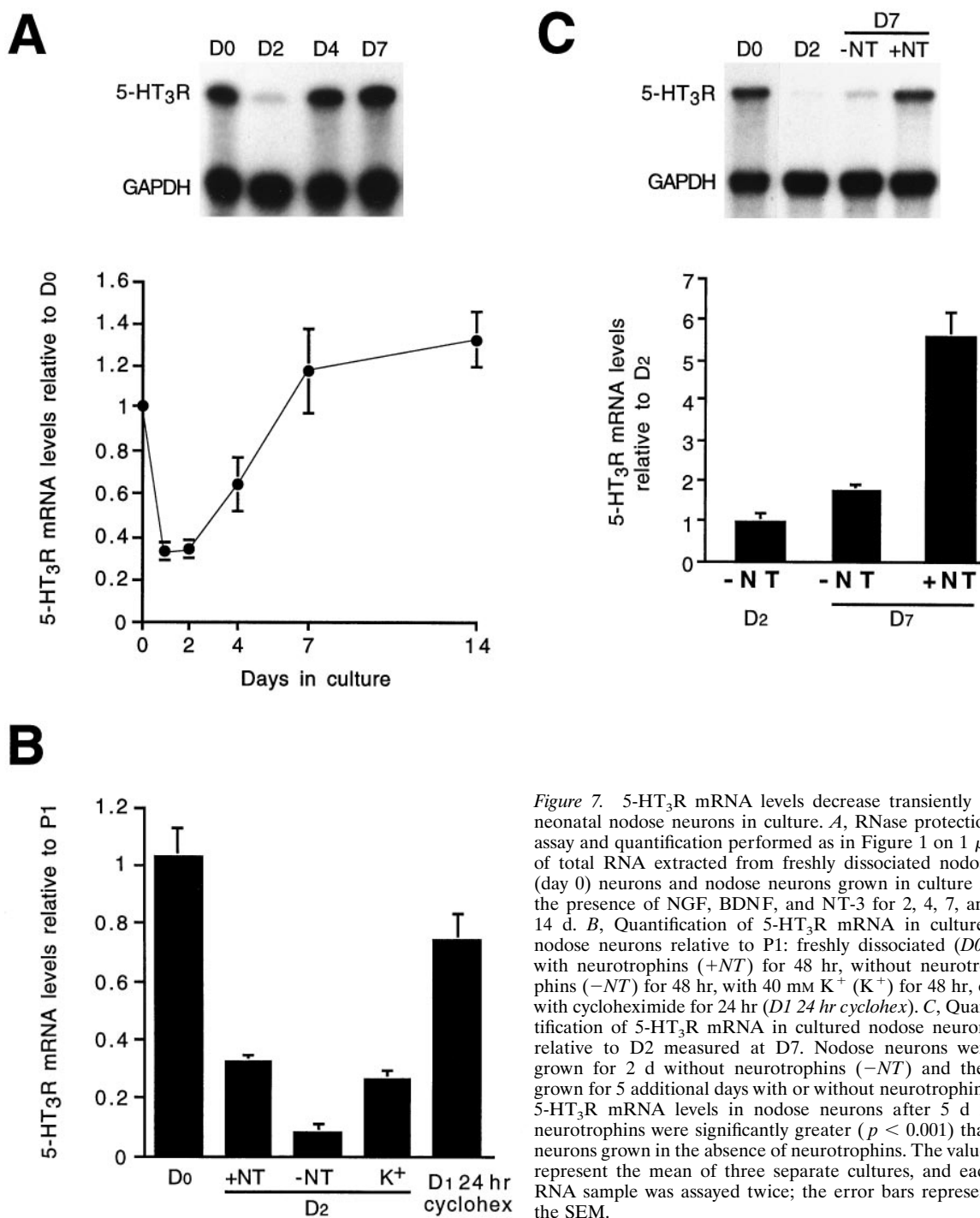


Figure 7. 5-HT₃R mRNA levels decrease transiently in neonatal nodose neurons in culture. *A*, RNase protection assay and quantification performed as in Figure 1 on 1 μ g of total RNA extracted from freshly dissociated nodose (day 0) neurons and nodose neurons grown in culture in the presence of NGF, BDNF, and NT-3 for 2, 4, 7, and 14 d. *B*, Quantification of 5-HT₃R mRNA in cultured nodose neurons relative to P1: freshly dissociated (*D0*), with neurotrophins (+*NT*) for 48 hr, without neurotrophins (*-NT*) for 48 hr, with 40 mM K⁺ (K⁺) for 48 hr, or with cycloheximide for 24 hr (*D1 24 hr cyclohex*). *C*, Quantification of 5-HT₃R mRNA in cultured nodose neurons relative to D2 measured at D7. Nodose neurons were grown for 2 d without neurotrophins (*-NT*) and then grown for 5 additional days with or without neurotrophins. 5-HT₃R mRNA levels in nodose neurons after 5 d in neurotrophins were significantly greater ($p < 0.001$) than neurons grown in the absence of neurotrophins. The values represent the mean of three separate cultures, and each RNA sample was assayed twice; the error bars represent the SEM.

levels, like that for nAChR transcripts (Mandelzys et al., 1994), is not dependent on preganglionic innervation; this suggests that neither electrical activity nor factors derived from the preganglionic nerve terminals stimulate 5-HT₃R expression in these neurons.

Surprisingly, we observed a significantly greater increase in 5-HT₃R gene expression when neonatal SCG neurons develop in culture as compared with when they develop *in vivo*: after 1 week in culture 5-HT₃R mRNA levels increase ninefold versus a threefold increase over the same time period *in vivo*. The greater stimulation of 5-HT₃R expression in cultured SCG neurons presumably results from the removal of an inhibitory influence

present *in vivo*, the addition of stimulatory factors to the cultures, or a combination of both. The greater increase in 5-HT₃R expression in cultured SCG neurons cannot be explained by changes in *trkA* or p75 receptor expression (M. R. and E. C., unpublished results).

When neonatal nodose neurons develop in culture in the presence of neurotrophins, 5-HT₃R mRNA levels drop by 70% in 24 hr, begin to increase after 48 hr, and are above initial levels after 1 week. This relatively rapid biphasic change in 5-HT₃R mRNA levels was unexpected because we did not observe a similar change in SCG neurons or with nodose neurons developing *in vivo*. We show that this increase in 5-HT₃R expression after 48 hr

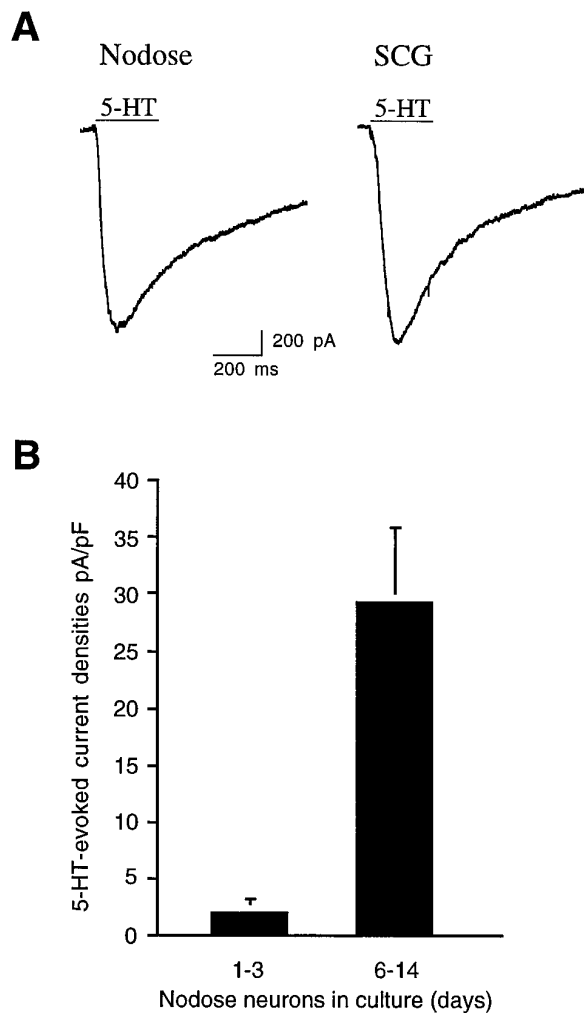


Figure 8. Nodose and SCG neurons express 5-HT-evoked currents. 5-HT-evoked currents were recorded from P1 nodose and SCG neurons grown in culture. *A*, Examples of 5-HT-evoked currents from D6 nodose and D7 SCG neurons. *B*, Mean 5-HT-evoked current densities (pA/pF) in nodose neurons grown for 1–3 d ($n = 36$) and 6–14 d ($n = 25$); the error bars represent the SEM.

in culture is mediated by neurotrophins. The increase in 5-HT₃R expression cannot be explained by changes in neurotrophin receptor expression by nodose neurons over this time in culture (D. W. and E. C., unpublished results). Because neurotrophins are released from non-neuronal cells in degenerating peripheral nerve stumps after axotomy (Heumann et al., 1987; Raivich et al., 1991), as well as from target tissues, this mechanism could ensure that, after a brief delay (24 hr), regenerating nodose axons will continue to express 5-HT₃R. Conceivably, neurotrophins also play a role in regulating 5-HT₃R expression in SCG neurons.

The initial rapid drop in 5-HT₃R mRNA levels is likely attributable to the effects of axotomy, because the axons of these neurons are cut when they are placed in culture. We show that cutting the peripheral branch of nodose axons in neonatal animals results in a rapid 60% decrease in 5-HT₃R mRNA levels in 24 hr. The decrease we observed in cultured neurons is greater than that in axotomized neurons *in vivo*, presumably because in cultured nodose neurons both the central and peripheral axons were cut. This indicates that the high 5-HT₃R transcript levels in developing nodose neurons are actively maintained by mechanisms that

depend on intact innervation, possibly involving target-derived factors, similar to what has been shown for nAChRs in chick ciliary neurons (Jacob and Berg, 1987; Levey et al., 1995; Levey and Jacob, 1996). Similar decreases in mRNA and protein levels have been reported for a number of neuropeptides and their receptors after axotomy of sensory neurons, and the decrease of many of these proteins can be prevented by target-derived factors (Herdegen and Zimmerman, 1994; Hökfelt et al., 1994a,b). However, we show that the rapid drop in 5-HT₃R mRNA levels in cultured nodose neurons is not prevented by neurotrophins, suggesting that cutting the axons may initiate events that actively decrease 5-HT₃R mRNA levels in nodose neurons.

Our results on culturing nodose neurons in elevated K⁺ indicate that chronic depolarization and the resulting increase in intracellular Ca²⁺ do not prevent the drop in 5-HT₃R mRNA levels. These experiments suggest that the rapid drop in 5-HT₃R mRNA levels in cultured nodose neurons, and presumably after axotomy *in vivo*, is not a result of Ca²⁺-dependent second messenger pathways. When we treat nodose cultures with cycloheximide, a protein synthesis inhibitor, the initial decrease in 5-HT₃R mRNA levels in large part is prevented, suggesting that this decrease is an active process that requires *de novo* protein synthesis.

Our results indicate that the biphasic change in 5-HT₃R mRNA levels in cultured nodose neurons represents the net effect of two separate processes that regulate gene expression: one triggered by axotomy that decreases 5-HT₃R mRNA levels and the other mediated by neurotrophins that increase these levels. Because the drop occurs during the first 24 hr, even in the presence of neurotrophins, the events initiated by axotomy in nodose neurons must outweigh the stimulatory effects of neurotrophins but are short-lived. We did not observe any decrease in 5-HT₃R mRNA levels in cultured SCG neurons. One possible explanation is that the stimulatory effects of neurotrophins on sympathetic neurons override the effects of axotomy. A second possibility is that sympathetic neurons differ from sensory neurons in their response to axotomy.

To address whether changes in 5-HT₃R gene expression affect the appearance of functional 5-HT₃Rs, we measured 5-HT-evoked currents on nodose and SCG neurons in culture. Our electrophysiological results indicate that changes in 5-HT₃R mRNA levels correlate well with the appearance of functional 5-HT₃R on these neurons. For nodose neurons, after 1–3 d in culture when 5-HT₃R mRNA levels are low, we found that few neurons had detectable 5-HT-evoked currents, whereas 6–14 d later >80% had detectable 5-HT-evoked currents. Similar results were observed for SCG neurons in culture. Our results demonstrate that changes in 5-HT₃R gene expression directly reflect changes in the appearance of functional receptors.

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