

Developmental Expression of the TTX-Resistant Voltage-Gated Sodium Channels Na_v1.8 (SNS) and Na_v1.9 (SNS2) in Primary Sensory Neurons

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The development of neuronal excitability involves the coordinated expression of different voltage-gated ion channels. We have characterized the expression of two sensory neuron-specific tetrodotoxin-resistant sodium channel α subunits, Na_v1.8 (SNS/PN3) and Na_v1.9 (SNS2/NaN), in developing rat lumbar dorsal root ganglia (DRGs). Expression of both Na_v1.8 and Na_v1.9 increases with age, beginning at embryonic day (E) 15 and E17, respectively, and reaching adult levels by postnatal day 7. Their distribution is restricted mainly to those subpopulations of primary sensory neurons in developing and adult DRGs that give rise to unmyelinated C-fibers (neurofilament 200 negative). Na_v1.8 is expressed in a higher proportion of neuronal profiles than Na_v1.9 at all stages during development, as in the adult. At E17, almost all Na_v1.8-expressing neurons also express the high-affinity NGF receptor

TrkA, and only a small proportion bind to IB4, a marker for c-ret-expressing (glial-derived neurotrophic factor-responsive) neurons. Because IB4 binding neurons differentiate from TrkA neurons in the postnatal period, the proportion of Na_v1.8 cells that bind to IB4 increases, in parallel with a decrease in the proportion of Na_v1.8–TrkA co-expressing cells. In contrast, an equal number of Na_v1.9 cells bind IB4 and TrkA in embryonic life. The differential expression of Na_v1.8 and Na_v1.9 in late embryonic development, with their distinctive kinetic properties, may contribute to the development of spontaneous and stimulus-evoked excitability in small diameter primary sensory neurons in the perinatal period and the activity-dependent changes in differentiation they produce.

Key words: sodium channels; TTXr; dorsal root ganglia (DRG); development; sensory neurons; growth factors

Primary somatosensory neurons show three specific temporal patterns of action potential firing in development (Fitzgerald, 1987; Meister et al., 1991; Fitzgerald and Fulton, 1992). During the period of neurite outgrowth in early embryonic development, dorsal root ganglion (DRG) neurons are electrically inexcitable (Fitzgerald, 1987). This is followed by a period of low-frequency spontaneous activity at embryonic day (E) 16–E20, largely coinciding with peripheral target innervation (E14–E19) (Fitzgerald, 1987; Meister et al., 1991; Fitzgerald and Fulton, 1992). Finally, after the formation of central synapses and peripheral terminals, the spontaneous activity is replaced by higher frequency sensory-evoked activity at which time the neurons adapt to a more differentiated pattern of excitability (Fitzgerald, 1987; Meister et al., 1991). This activity contributes to selective gene expression (Dietzel, 1995; Spitzer et al., 1995; Fields, 1996) promoting neuronal differentiation of both primary sensory (Gu and Spitzer, 1995; Brose et al., 1998) and spinal cord neurons (Holliday and Spitzer, 1990).

The sodium currents of DRG neurons are divided into two

types on the basis of their sensitivity to tetrodotoxin (TTX) (Waxman et al., 1999). All primary sensory neurons express a low activation threshold, fast-inactivating, TTX-sensitive (TTXs) current (Kostyuk et al., 1981; Caffrey et al., 1992; Catterall, 1992; Roy and Narahashi, 1992). Small DRG neurons also display a number of high activation threshold, slowly activating and inactivating TTX-resistant (TTXr) currents (TTX-R1, R2, R3) (McLean et al., 1988; Roy and Narahashi, 1992; Elliott and Elliott, 1993; Arbuckle and Docherty, 1995; Rush et al., 1998). In DRG neurons, the TTXs current is mediated by one or more of the following sodium channel α -subunits known, according to the new standardized nomenclature (Goldin et al., 2000), as Na_v1.1 (rat I), Na_v1.2 (rat IIa), Na_v1.3 (rat III), Na_v1.7 (PN-1/hNE) (Toledo-Aral et al., 1997; Cummins et al., 1998), and Na_v1.6 (NaCh6/SCN8A/PN4) (Black et al., 1996; Waxman et al., 1999; Tzoumaka et al., 2000), whereas the TTXr current is mediated by two sensory neuron-specific sodium channels, Na_v1.8 (SNS/PN3) (Akopian et al., 1996; Sangameswaran et al., 1996) and Na_v1.9 (SNS2/NaN) (Dib-Hajj et al., 1998b, 1999; Tate et al., 1998; Fjell et al., 2000). The ratio of the TTXs/TTXr current will affect excitability by influencing action potential kinetics, including activation threshold, rate of rise, peak amplitude, duration, and capacity to fire repetitively (Rush et al., 1998). In the adult DRG, Na_v1.8 is expressed in both A- and C-fiber neurons (Amaya et al., 2000; Renganathan et al., 2000), whereas Na_v1.9 is preferentially expressed in C-fiber neurons (Tate et al., 1998; Amaya et al., 2000; Fjell et al., 2000). Both channels are strongly implicated in the molecular mechanisms of nociception (Okuse et al., 1997; McCleskey and Gold, 1999).

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Na_v1.3 has a developmentally regulated pattern of expression, peaking at E17 and downregulated by birth (Waxman et al., 1994; Felts et al., 1997). The expression of both Na_v1.6 and Na_v1.7 increase postnatally, whereas the Na_v1.2 and Na_x (NaG) α -subunits, which are expressed in large DRG neurons, are constitutively expressed throughout development (Felts et al., 1997). We have now investigated the onset and pattern of expression of the TTXr sodium channels Na_v1.8 and Na_v1.9 in late embryonic and early postnatal periods.

MATERIALS AND METHODS

Time-mated pregnant Sprague Dawley rats were used for all procedures in accordance with the United Kingdom Animals (Scientific Procedures) Act (1986) and following Massachusetts General Hospital animal care guidelines. Rats were designated postnatal day (P) 0 on the day of parturition.

Tissue preparation. For embryonic tissue, time-mated pregnant rats were terminally anesthetized with CO₂ and exsanguinated, and the embryos were harvested and collected in cold PBS. For Northern blot analysis, all lumbar DRGs were removed from two rat litters per age category (E15, E17, E19, and P0) and processed for RNA extraction. For immunohistochemistry, the L4 and L5 DRGs were dissected from overnight post-fixed [4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4] whole embryos (E15, E17, and E19). DRGs (L4, L5) were dissected from neonatal (P0 and P7) and adult animals after they were killed with intraperitoneal injection of sodium pentobarbital (200 mg/kg) (Duphar) and transcardiac perfusion with 4% paraformaldehyde in 0.1 M PB. DRGs were post-fixed overnight and cryoprotected with 20% sucrose for 3 hr.

Northern blot analysis. Total RNA was extracted from homogenized tissue samples by acid phenol extraction according to the technique of Chomczynski and Sacchi (1987). The RNA (15 μ g per sample) was separated on 1.5% formaldehyde–agarose gels and blotted onto Hybond N+ nylon membranes. RNA markers ranging from 0.24 to 9.5 kb (Life Technologies, Gaithersburg, MD) were used to determine transcript size. Filters were hybridized in a solution containing 50% formaldehyde, 5 $\times$ SSC, 5 $\times$ Denhardt's solution, 1% SDS, and 100 μ g/ml sheared herring sperm DNA at 42°C. The filters were washed in 0.1 $\times$ SSC and 0.1% SDS at 42°C. Na_v1.8 (650 bp), Na_v1.9 (500 bp), and cyclophilin (240 bp) probes were prepared as described in Costigan et al. (1998) and Amaya et al. (2000). A 50 ng sample of each probe was radiolabeled by incorporation of 50 μ Ci of [³²P]dCTP and separated from unincorporated nucleotides on Sephadex G-50 columns. At least two independent Northern blots obtained from RNA that was extracted from different pools of animals were used for each observation.

Immunocytochemistry. For colocalization of Na_v1.8 or Na_v1.9 with IB4 (isolectin B4 from *Griffonia simplicifolia*) or the A-fiber marker NF200, sections were blocked for 1 hr at room temperature with PBS containing 0.3% Triton X-100 (Tx-100; Sigma, St. Louis, MO) and 20% normal goat serum (Vector Laboratories, Burlingame, CA). Embryonic tissue was pretreated with 3% hydrogen peroxidase (Sigma) for 30 min to reduce endogenous peroxidase background. Primary antibody incubations with rabbit polyclonal antibodies against Na_v1.8 or Na_v1.9 (1:500 and 1:750 dilutions, respectively) (Amaya et al., 2000) and the mouse monoclonal antibody NF200 (1:200; Sigma) or biotin-conjugated IB4 (40 μ g/ml; Sigma) were performed in PBS containing 0.1% Tx-100 overnight at 4°C. Secondary antibody incubation with CY3-conjugated anti-rabbit IgG (1:300; Jackson ImmunoResearch, West Grove, PA) and FITC-conjugated anti-mouse IgG (1:200; Vector Laboratories) or FITC-conjugated avidin (1:200; Vector Laboratories) was performed in PBS containing 0.1% Tx-100 for 3 hr at room temperature after three 10 min washes with PBS.

For colocalization of Na_v1.8 or Na_v1.9 and TrkA, sections were immunostained using red-direct tyramide signal amplification (TSA-red; NEN, Boston, MA). The technique enables double labeling with two antibodies raised in the same host animal without cross-reactivity (Michael et al., 1997). Sections were incubated with primary antibodies Na_v1.8 (1:1500) or Na_v1.9 (1:3000) in TNB buffer (0.1 M Tris-buffered saline, pH 7.4, containing 1% blocking reagent) overnight at 4°C, followed by three 5 min washes in wash buffer (0.1 M Tris, pH 7.5, 0.15 M NaCl, 0.05% Tween 20) and secondary antibody incubation with biotin-conjugated anti-rabbit antiserum (1:100; Vector Laboratories) in TNB buffer for 2 hr at room temperature. After washing (three 5 min washes in wash buffer), sections

were incubated with streptavidin-HRP (1:100 in TNB buffer) followed by incubation with fluorophore tyramide (1:50 in amplification diluent) for 7–10 min at room temperature. After three washes in TSA wash buffer, sections were incubated with TrkA (1:750, generously provided by Louis Reichardt, University of California at San Francisco) in PBS containing 0.1% Tx-100 at 4°C overnight and processed as for secondary antibody incubation as mentioned above, except using FITC-conjugated anti-rabbit secondary antibody (1:200, Vector Laboratories). Fluorescent images were captured by use of a digital (Nikon Spot) camera, except where confocal microscopy (Leica, Nussloch, Germany) was used for high magnification of embryonic tissue images.

For comparative purposes, duplicate slides were prepared by collecting consecutive 10 μ m sections on sequential slides, allowing different antibody combinations to be tested on the same DRG. Sections collected on an individual slide were equally spaced throughout the ganglion (L4) as follows: every 40 μ m for E15–E19, 100 μ m for P0, and 1200 μ m for P7–adult. The first section to be placed on the first slide was chosen randomly in each case.

Data analysis. Na_v1.8 and Na_v1.9 colocalization with each neuronal marker was performed by computer analysis using Freehand (Macromedia), which enabled positively immunostained cell profiles (cells with a clearly identifiable nucleus) for each antibody to be marked and counted, and overlaying the two images was used to count double-labeled cells. Total cell profiles were determined by enhancing the brightness of the green (FITC) image and counting all cell profiles not previously marked as being single or double immunostained. For analysis only of the E17 age category, the image of the entire DRG was divided into four equal quarters, and one quarter was counted at random. The first section to be photomicrographed for each age category was selected at random, and four to six sections evenly spaced throughout the DRG were counted ($n = 5$ per age category).

Change in expression of the neuronal markers (TrkA, IB4, and NF200) and Na_v1.8 or Na_v1.9 in the DRG during development is presented as the proportion of positive profiles per total cell profiles. This was determined as follows: the percentage of positive immunostained profiles per section was averaged for each animal (5–6 sections per animal). Five separate averages ($n = 5$ animals) were expressed as the mean and SEM to give the final values. No statistically significant variance was found by the ANOVA paired test of the values for TrkA, IB4, and NF200, when expressed as percentage of total profiles, between the two data sets obtained from independent experiments (Na_v1.8 or Na_v1.9 at different ages). Therefore, these essentially identical data sets were pooled (Table 1, % neuronal marker in the total cell population).

RESULTS

Developmental expression of Na_v1.8 and Na_v1.9 in the DRG

Changes in Na_v1.8 and Na_v1.9 mRNA and protein expression in rat lumbar DRGs were analyzed at late embryonic (E15, E17, E19) and early neonatal stages (P0) of development.

An 8.0 kb Na_v1.8 mRNA transcript was detected at low levels in lumbar DRGs at E15. At this stage, no Na_v1.9 transcript could be seen. At E17, however, both an Na_v1.8 and an Na_v1.9 mRNA transcript (7.5 kb) were detectable in the DRG (Fig. 1). A second alternatively spliced Na_v1.8 transcript of 9.5 kb, similar to that previously described in the adult (Amaya et al., 2000) could be seen by E17 and at all later stages, which was not present at E15. The relative amount of both the Na_v1.8 and Na_v1.9 mRNA transcripts increased from E17 to P0 (Fig. 1).

The proportion of DRG neuronal profiles expressing Na_v1.8 and Na_v1.9 protein was assessed at developmental stages E17, P0, P7, and P21, as well as in the adult (Amaya et al., 2000). While a few Na_v1.8-labeled profiles could be seen in E15 DRGs (data not shown), Na_v1.9-labeled profiles could not be detected until E17, which indicates a coordinated temporal pattern of expression of messenger mRNA with protein expression for both Na_v1.8 and Na_v1.9. At E17, ~25% of DRG neurons expressed Na_v1.8, and this increased to ~50% (adult levels) by P7 (Fig. 2A). The proportion of Na_v1.9-positive neuronal profiles increased from

Table 1. The expression of Na_v1.8 and Na_v1.9 within sensory neuronal subtypes in the DRG with increasing development

Neuronal marker	Age category	% neuronal marker in total cell population	% Na _v 1.8 in neuronal marker population	% Na _v 1.9 in neuronal marker population
TrkA	E17	79.5 (±1.0)	78.8 (±1.3)	55.0 (±3.0)
	P0	78.6 (±0.6)	63.0 (±3.5)	48.9 (±2.5)
	P7	60.2 (±3.2)	51.1 (±2.0)	43.7 (±2.6)
	P21	45.3 (±1.8)	41.8 (±2.6)	43.4 (±3.5)
	Adult	42.0	49.2 (±5.3)	45.5 (±6.0)
IB4	E17	15.0 (±0.8)	13.4 (±1.9)	44.1 (±3.1)
	P0	28.2 (±0.8)	21.4 (±2.8)	64.8 (±3.8)
	P7	42.3 (±1.2)	31.5 (±1.6)	60.5 (±1.3)
	P21	39.3 (±1.2)	45.2 (±1.8)	59.9 (±1.7)
	Adult	40.0	55.0 (±6.7)	59.8 (±6.0)
NF200	E17	21.2 (±1.7)	11.0 (±1.4)	0.83 (±2.2)
	P0	35.7 (±1.0)	14.5 (±1.6)	8.62 (±2.0)
	P7	42.4 (±1.5)	12.1 (±2.6)	6.64 (±1.4)
	P21	47.0 (±1.9)	13.0 (±1.8)	2.64 (±0.5)
	Adult	40.0	18.4 (±2.7)	0 (±0)

The change in expression of the neuronal markers (TrkA, IB4, and neurofilament NF200) in the DRG during development is represented as a proportion of positive profiles from the total cell profile population. The change in the distribution of Na_v1.8 and Na_v1.9 expression within each neuronal subtype is represented as a proportion of Na_v1.8 and Na_v1.9 positive profiles within the total neuronal marker profile population. Each value represents the mean percentage of positive profiles from 10 animals per age category for the % neuronal marker column, and 5 animals per age group for the Na_v1.8 and Na_v1.9 columns, with the SEM between animals in parentheses.

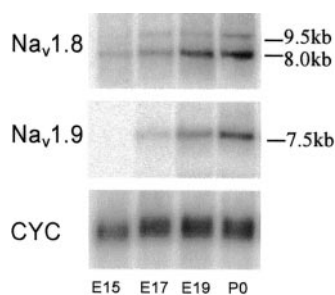


Figure 1. The developmental regulation of Na_v1.8 and Na_v1.9 expression in embryonic DRG. Northern blot analysis of Na_v1.8 (*top*) and Na_v1.9 (*middle*) α -subunit mRNA transcripts in lumbar DRG between developmental ages E15 and P0. Cyclophilin mRNA (*CYC*, *bottom*) is a loading control.

~20% at E17 to ~40% (adult levels) at P7 (Fig. 2*A*). At all developmental ages, significantly fewer neurons express Na_v1.9 than Na_v1.8 (E17 to P0, $p < 0.01$; P7 to adult, $p < 0.005$; ANOVA test) (Fig. 2*A*).

Developmental expression of Na_v1.8 and Na_v1.9 in neuronal subpopulations

Co-expression of Na_v1.8 and Na_v1.9 with markers of different neuronal subpopulations was studied in the developing DRG. These included TrkA (to identify NGF-responsive, small and medium diameter, peptide-containing neurons) (McMahon et al., 1994; Averill et al., 1995; Molliver et al., 1995), IB4 [to identify nonpeptide containing, glia-derived neurotrophic factor (GDNF) responsive small diameter neurons] (Plenderleith et al., 1988; Plenderleith and Snow, 1993), and neurofilament (NF200; to identify large diameter cells with myelinated axons) (Price, 1985; Lawson and Waddell, 1991; Lawson et al., 1993).

Although the proportion of NF200-positive profiles increased from 21% at E17 to adult levels by P7 (Table 1), the proportion of Na_v1.8-expressing neurons that co-expressed this marker was low at all stages of development (11% at E17 and 13% at P7), which is comparable with adult levels of 18% (Fig. 2*B*, Table 1). A very

small proportion of Na_v1.9-expressing cells (<9%) were found to co-express NF200 at all ages, indicating that this ion channel, as in the adult (Tate et al., 1998; Amaya et al., 2000; Fjell et al., 2000), is predominantly found in cells destined to have unmyelinated axons.

At E17, Na_v1.8 was found mainly in TrkA-positive neurons (~80%) with only 13% IB4-positive cells. The proportion of Na_v1.8 cells that were IB4-positive increased postnatally, reaching adult levels of 45% at P21. This was accompanied by a parallel decrease in the proportion of Na_v1.8-positive cells also expressing TrkA, so that eventually Na_v1.8 is equally represented in both sets of neurons (Fig. 2*B*, Table 1). As reported previously, the proportion of TrkA-positive cells in the total population decreased from 80% at E17 to 45% at P21, whereas that of IB4 increased from 15 to 40% over this time (Table 1) (Bennett et al., 1996).

Typical examples of Na_v1.8 co-expression with TrkA are shown in Figure 3, illustrating the transition from the majority of Na_v1.8-expressing cells being TrkA-positive at E17 to ~40% colocalization at P21. As in the adult (Amaya et al., 2000), all medium diameter Na_v1.8-positive cells were TrkA-positive, although this does not hold true for the reverse situation; some medium TrkA-positive cells did not co-express Na_v1.8. In contrast to the Na_v1.8 population, many Na_v1.9-positive neurons were also IB4-positive at E17 (Fig. 2*B*, Table 1), although the overall IB4-positive population is low at this stage (15%). The proportion of Na_v1.9–IB4 co-expressing cells reached adult levels at P0, despite the fact that the upregulation of IB4 to adult levels is only reached at P7 (Table 1). Figure 4 shows representative illustrations of the pattern of colocalization of Na_v1.9 with the three different markers at developmental ages E17, P0, and P7. As in the adult, all Na_v1.9 profiles possess a small diameter at all ages, unlike Na_v1.8 in which ~20% of the cells have medium-sized diameters.

DISCUSSION

The expression of voltage-gated ion channels is fundamental to neuronal excitability and, therefore, to spontaneous and stimulus-evoked activity in primary sensory neurons. The regulation of ion channel expression may be influenced by both intrinsic develop-

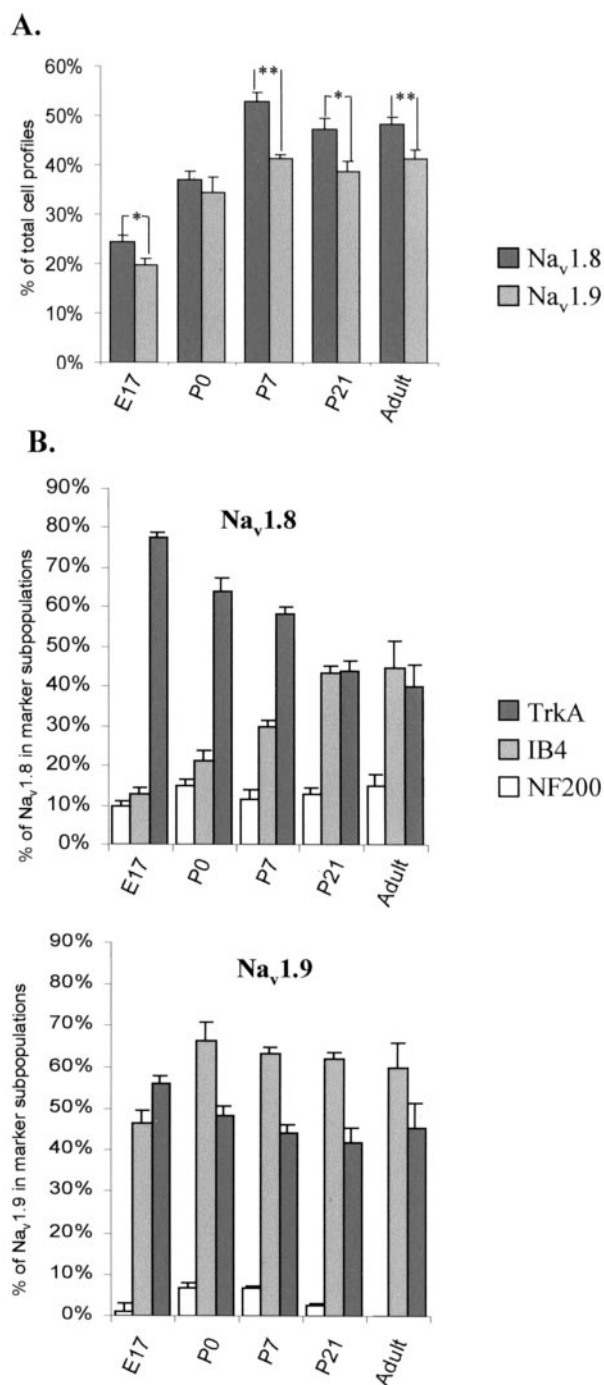


Figure 2. Quantitative analysis of Na_v1.8 and Na_v1.9 protein expression levels through embryonic and neonatal development. *A*, The percentage of Na_v1.8- or Na_v1.9-positive cell profiles as a proportion of the total number of DRG neuronal profiles in the developing DRG. Error bars represent SEM. (* $p < 0.01$, ** $p < 0.001$, for values for Na_v1.8 vs Na_v1.9; ANOVA paired test). Levels of SNS and SNS2 protein expression are significantly different ($p = 0.001$; ANOVA) from E17 to P7, compared with adult Na_v1.8 and Na_v1.9 expression. *B*, The proportion of the Na_v1.8- and Na_v1.9-positive profiles expressed in neuronal subpopulations expressing NF 200, TrkA, and IB4 binding, with increasing development.

mental signals, such as time-dependent changes in transcription factors, and extrinsic developmental events, such as changes in neurotrophic factor expression, so that the distribution of ion channels may differ within the developing and mature nervous

systems. In this study, we characterized the developmental expression of two TTXr sensory neuron-specific sodium channels, Na_v1.8 (SNS) and Na_v1.9 (SNS2), within defined neuronal populations of the DRG.

Na_v1.8 is the first TTXr sodium channel to be expressed, at E15, before both its larger putative alternatively spliced transcript and Na_v1.9, which becomes detectable only at E17. The distribution of Na_v1.9 in small C-fiber neurons (Dib-Hajj et al., 1998a; Amaya et al., 2000) may explain its later onset of expression compared with Na_v1.8, because C-fiber cells develop later than larger A-fiber neurons (Altman and Bayer, 1984). The level of expression of both Na_v1.8 and Na_v1.9 increased with increasing development, reaching adult levels by P7, with a higher proportion of total neuronal profiles expressing Na_v1.8 than Na_v1.9 at all stages in development. Na_v1.9, as in the adult, is effectively restricted to small diameter sensory neurons at all stages, whereas Na_v1.8 is expressed mainly in small diameter DRG neurons but in some medium-sized NF200-positive neurons as well. By P21, both Na_v1.8 and Na_v1.9 exhibit similar distribution patterns to that found in the adult (Amaya et al., 2000).

Na_v1.8 and Na_v1.9 appear to be regulated by distinct mechanisms

Despite similar and largely overlapping distribution patterns of both the TTXr channels in small diameter adult sensory neurons (Amaya et al., 2000), the expression of Na_v1.8 and Na_v1.9 within defined neuronal subpopulations differed substantially in early development. At E17, Na_v1.8 is expressed mostly in TrkA-positive, NGF-responsive neurons, with little expression in the IB4-binding population. Since TrkA neurons differentiate into IB4-positive, c-ret-expressing GDNF-responsive neurons at the time of birth (Bennett et al., 1996; Molliver and Snider, 1997; Molliver et al., 1997), the proportion of Na_v1.8 colocalization within this IB4-binding population increases proportionally, with a simultaneous relative decrease in TrkA–Na_v1.8-expressing neurons. In contrast, at the onset of Na_v1.9 expression at E17, approximately half of the Na_v1.9-positive population co-express IB4, although only a very small minority of total DRG neurons are IB4-positive at this time. The remaining half colocalize with TrkA, which is the neuronal population that constitutes the vast majority of small diameter sensory neurons at this stage (Bennett et al., 1996; Molliver and Snider, 1997). This difference in the embryonic distribution pattern of Na_v1.8 and Na_v1.9 suggests that the cellular pattern of expression of each channel is likely to be controlled by distinct promoter regions, responding to different transcription factors in the late embryonic period.

In the adult, there is evidence to suggest an NGF- and GDNF-dependent regulation of Na_v1.8 (Hinson et al., 1997; Dib-Hajj et al., 1998a; Fjell et al., 1999a,b) and Na_v1.9 (Fjell et al., 1999c; Cummins et al., 2000), respectively, although both channels are expressed by TrkA and IB4-positive cells (Amaya et al., 2000). At the time of IB4-positive (GDNF-responsive) cell differentiation (at E17), these neurons express Na_v1.9 before Na_v1.8. The onset of Na_v1.9 expression at E17 coincides, moreover, with the release of GDNF from Schwann cells between E14 and E16 (Wright and Snider, 1996) (Fig. 5). Na_v1.8 expression at E15 coincides with the beginning of A- and C-fiber distal target innervation (Molliver and Snider, 1997). This suggests that Na_v1.8 may be regulated by target-derived neurotrophic factors (Dib-Hajj et al., 1998a; Fjell et al., 1999a,b,c) (Fig. 5) because NGF expression begins in the skin at time of innervation (Thoenen et al., 1988; Elkabes et al., 1994). Both Na_v1.8 and Na_v1.9 are downregulated in the adult by

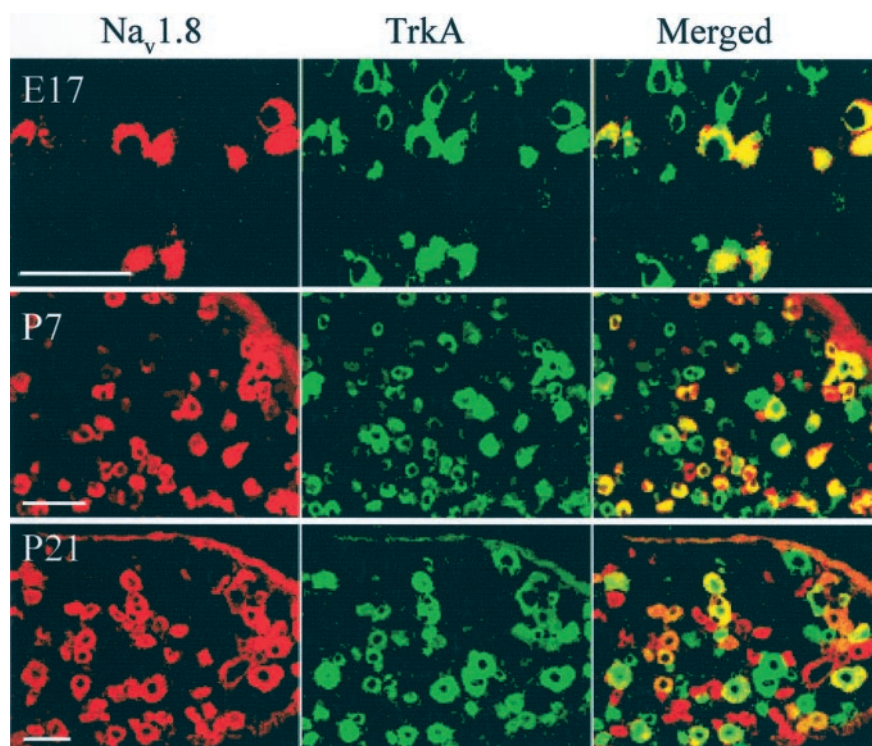


Figure 3. Distribution of $\text{Na}_v1.8$ changes with phenotypic changes in the developing DRG. Double immunocytochemistry of $\text{Na}_v1.8$ labeled (red), TrkA labeled (green), and composite image (far right panel) showing double labeled (yellow) DRG cells at specific developmental ages. Scale bars, 50 μm .

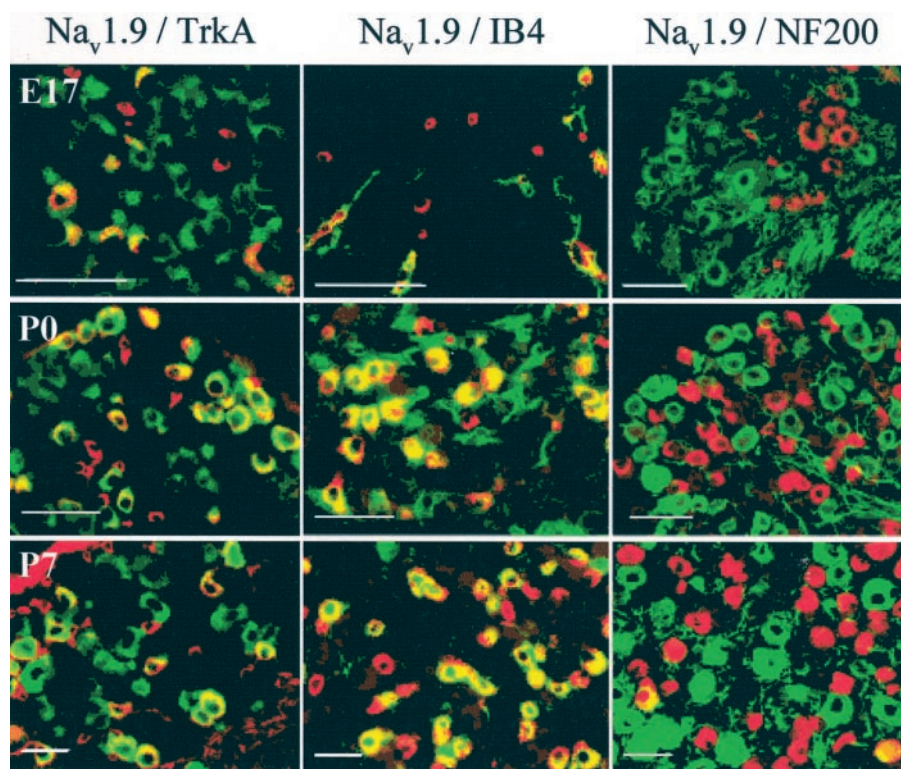


Figure 4. Distribution of $\text{Na}_v1.9$ in subpopulations of DRG neurons through development. Immunoreactivity of cells positive for $\text{Na}_v1.9$ (red), marker population (green), or both (yellow) (composite image) in $\text{Na}_v1.9$ colocalization studies with TrkA (left), IB4 (middle), and NF200 (right) in DRG neurons at developmental ages E17, P0, and P7. Scale bars, 50 μm .

peripheral axotomy (Dib-Hajj et al., 1998b; McCleskey and Gold, 1999) but not by rhizotomy (Sleeper et al., 2000), supporting the hypothesis of a regulatory mechanism arising from peripheral target-derived signals.

Role of $\text{Na}_v1.8$ and $\text{Na}_v1.9$ in sensory neurons during development

The earliest electrophysiological recordings of TTXr currents have been from E17 rat DRG neurons (Ogata and Tatebayashi,

1992), fetal mouse, and cultured fetal human DRG neurons (Orozco et al., 1988; Caviedes et al., 1990). When $\text{Na}_v1.8$ sodium channels are first expressed in rat lumbar DRG at E15, sensory neurons are electrically inexcitable (Fig. 5). However, the characteristic fast repriming kinetics of $\text{Na}_v1.8$ (Rush et al., 1998; Waxman, 1999; Waxman et al., 1999; Renganathan et al., 2000), which allows sustained action potential firing during prolonged depolarization, may mean that it contributes to the observed

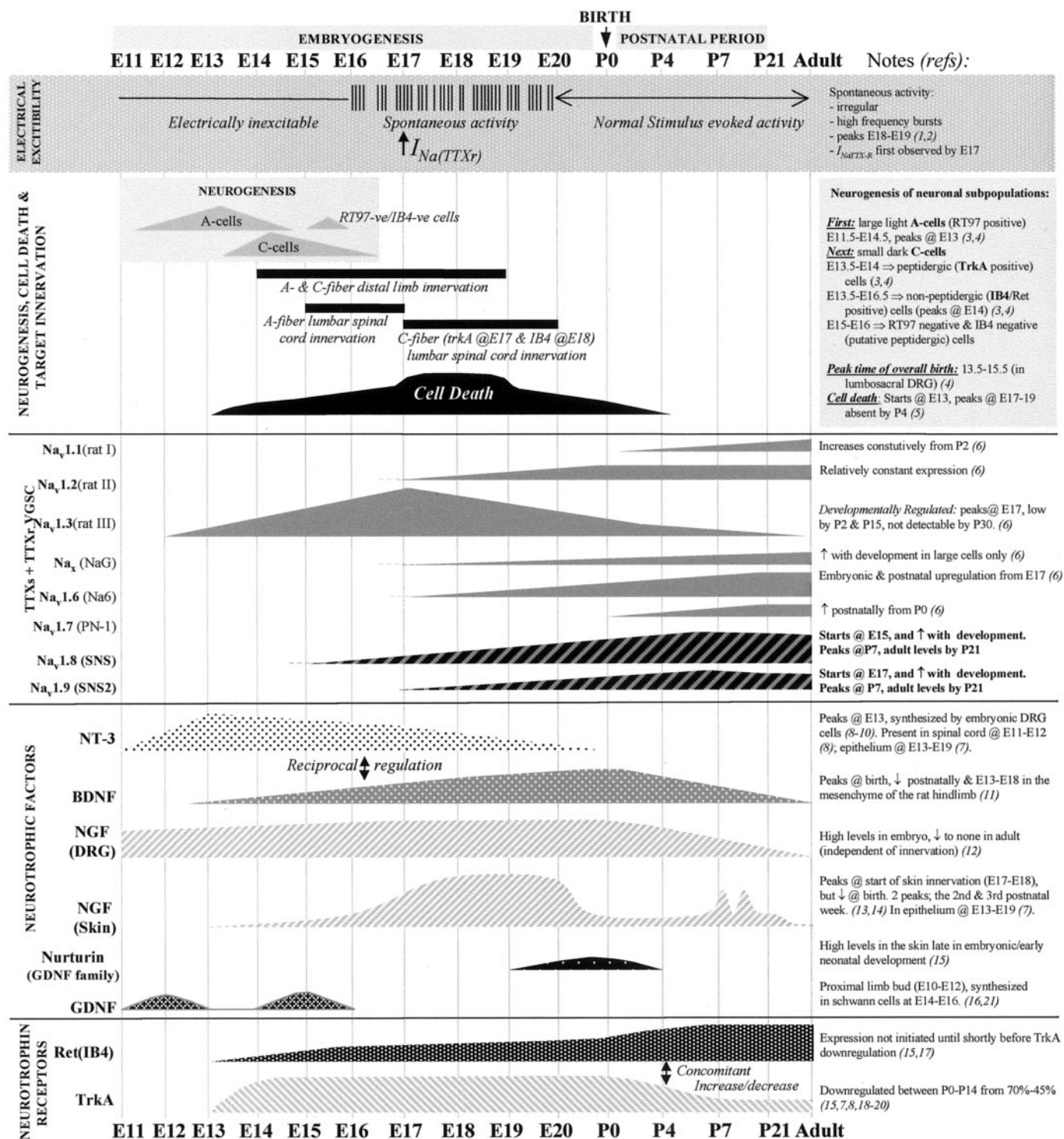


Figure 5. A summary of significant events defining rat DRG sensory neuron development. A schematic representation showing a time scale of developmentally regulated events of a typical DRG is illustrated from embryonic age 11 (E11) to postnatal age 21 (P21) and in adult animals (left to right of the diagram; not to scale). This is an attempt to correlate the major changes that occur in the developing DRG, with particular emphasis on acquisition of neuronal electrical excitability and changes in the expression of voltage-gated sodium channels (VGSCs). The expression of the sodium channels may be regulated by the developmental expression of specific neurotrophic factors. The top (shaded) panel illustrates the distinct patterns of electrical activity in the developing DRG, marking the period of developmentally regulated ectopic spontaneous discharge from E16 to E20, and the earliest detection of a TTX-resistant (TTXr) sodium current. The second panel outlines key developmental events, including the birth of specific neuronal subpopulations; the large light A-cell population (future A fibers) are born from E11.5 to E14.5, in advance of the birth of small, dark C-cell neurons (giving rise to C-fibers) which occurs later at E13.5–E16.5. A third unidentified subpopulation of putative nonpeptide-containing neurons (RT97-negative and IB4-negative) are generated between E14 and E15. Almost immediately after neuronal birth, the onset of axon outgrowth begins from E14 onward, followed by peripheral innervation of both A- and C-fibers simultaneously from E14 and central target innervation from E15 or E18 for A- and C-fibers, (Figure legend continues.)

spontaneous activity in DRG neurons between E16 and E20 (Fitzgerald, 1987; Meister et al., 1991; Fields, 1998). Although the role of this period of spontaneous activity remains unclear, it spans the period of peripheral target innervation (Jackman and Fitzgerald, 2000), C-fiber central target innervation and synaptogenesis (Jackman and Fitzgerald, 2000), and has been suggested to play an important role in promoting neuronal differentiation (Gu and Spitzer, 1997), activity-dependent regulation of specific genes (Holliday and Spitzer, 1990; Dutton et al., 1993; Fields, 1996, 1998), and the stabilization of synapses (Greensmith and Vrbova, 1996). Furthermore, the onset of expression of Na_v1.8 coincides with the period of time when sodium channel β 1-subunit mRNA is upregulated (Zur et al., 1995), which may contribute to the regulation and stability of the functional sodium channel (Catterall, 2000). However, as Na_v1.8 null mutant mice display no obvious evidence of developmental abnormalities (Akopian et al., 1999; Cummins et al., 1999), it is possible that Na_v1.8 contributes only slightly (Matsuda et al., 1978), if at all, to the differentiation of primary sensory neurons and the spinal neurons they innervate. A transgenic knockdown of Na_v1.9 and double knockouts will help establish whether TTXr sodium currents play a role in the maturation of the peripheral nervous system.

TTXr sodium currents have been shown to contribute to the initiation of action potential depolarization in immature neurons (Orozco et al., 1988), so expression of Na_v1.8 and Na_v1.9 in embryonic and neonatal sensory neurons may contribute to the capacity of these neurons to conduct electrical signals to the spinal cord. In the adult, the TTXr sodium currents in the peripheral terminals of nociceptors contribute to the regulation of the excitability of the terminals. Inflammatory mediators such as Prostaglandin E₂ (PGE₂), for example, appear to contribute to peripheral sensitization by a PKA-dependent phosphorylation of Na_v1.8, increasing the sodium current density (Jeftinija, 1994; England et al., 1996; Gold et al., 1996, 1998; Tanaka et al., 1998; Gold, 1999). The contribution of TTXr sodium channels to injury- and inflammatory-induced pain hypersensitivity at early developmental stages remains to be examined.

Reciprocal regulation of TTXr sodium channels and Na_v1.3

The onset of Na_v1.9 expression at E17 correlates with the start of embryonic Na_v1.3 sodium channel downregulation (Waxman et al., 1994; Felts et al., 1997). The opposite pattern occurs after adult peripheral nerve injury in which downregulation of both Na_v1.8 and Na_v1.9 is accompanied by a simultaneous upregulation of Na_v1.3 (Waxman et al., 1994; Rizzo et al., 1995; Black et al., 1997, 1999; Cummins and Waxman, 1997). Our data are in agreement with the findings by Black et al. (1997), suggesting reciprocal developmental and injury-induced regulation of Na_v1.8 and Na_v1.9 with Na_v1.3. Future studies directed toward the iso-

lation and functional analysis of transcriptional promoter regions from Na_v1.3, Na_v1.8, and Na_v1.9 genes may shed light on potential mechanisms that underlie this reciprocal regulation.

Conclusions

Figure 5 illustrates the relative timing of key events in the developing rat lumbar DRGs, including electrical activity, target innervation, exposure to growth factors, and sodium channel expression. This figure is an attempt to correlate the major changes that occur in the developing DRG, with particular emphasis on the link between the acquisition of neuronal electrical excitability and the expression of specific voltage-gated sodium channels (VGSCs). Expression of the VGSCs may be controlled by the developmental expression of specific neurotrophic factors that may regulate the differentiation of the neurons. Responsiveness of the neurons to these neurotrophic factors is contingent on expression of their cognate receptors. Key events, including birth of different subsets of neurons, natural cell death, axonal outgrowth, and peripheral and central target innervation are illustrated. The transcriptional regulation of the sodium channels, their expression only in particular subsets of neurons, their specific contribution to the electrical activity of immature neurons, and the consequence of this for differentiation is still poorly understood. Nevertheless, elucidation of the differential developmental patterns of expression of these channels, including Na_v1.8 and Na_v1.9, will provide a basis for beginning to understand the onset and maturation of excitability in primary sensory neurons.

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(Figure legend continued.) respectively. The middle panel shows changes in mRNA expression of known TTX-sensitive (TTXs) and the TTX-resistant (TTXr) voltage-gated sodium channels (VGSC) in the DRG, which may be correlated with developmentally regulated changes in neurotrophic factors (second panel from bottom) or changes in neuronal phenotype, which is indicated by a shift in the expression of specific neurotrophic receptors (bottom panel). Dotted lines indicate that expression at earlier ages has not yet been investigated. Arrows in the text denote an increase or decrease in expression, respectively. References are indicated by numbers on right side of figure: 1. Fitzgerald, 1987; 2. Fitzgerald and Fulton, 1992; 3. Jackman and Fitzgerald, 2000; 4. Altman and Bayer, 1984; 5. Coggeshall et al., 1994; 6. Felts et al., 1997; 7. Ernfor et al., 1992; 8. Ernfor et al., 1989; 9. ElShamy and Ernfor, 1996; 10. Schecterson and Bothwell, 1992; 11. Seibert and Shooter, 1993; 12. Constantinou et al., 1994; 13. Davis et al., 1987; 14. Rohrer et al., 1998; 15. Molliver et al., 1997b; 16. Alvares and Fitzgerald, 1999; 17. Bennett et al., 1996; 18. Phillips and Armanini, 1996; 19. Farinas et al., 1998; 20. Ehrhard and Otten, 1994; 21. Wright and Snider, 1996.

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