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Expression of sodium channels Na_v1.2 and Na_v1.6 during postnatal development of the retina

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Abstract

During the second and third postnatal weeks, there is a developmental switch from sodium channel isoform Na_v1.2 to isoform Na_v1.6 at initial segments and nodes of Ranvier in rat retinal ganglion cells. We used quantitative, real-time PCR to determine if the developmental appearance of Na_v1.6 channels is accompanied by an increase in steady-state level of Na_v1.6 mRNA in the retina. Between postnatal day 2 (P2) and P10, Na_v1.6 levels did not change, but between P10 and P19, there was an approximately 3-fold increase in Na_v1.6 transcript levels. This coincides with the appearance of Na_v1.6 channels in the retina and optic nerve. The steady-state level of Na_v1.2 mRNA also increased during this same period, which suggests that the rise in Na_v1.6 may be part of a general increase in sodium channel transcripts at about the time of eye-opening at P14. The results are consistent with a developmental increase in steady-state transcripts giving rise to a corresponding increase in sodium channel protein expression.

Keywords

retinal ganglion cell; sodium channels; quantitative PCR; axon initial segment; retina development

Introduction

Voltage-gated sodium (Na_v) channels accumulate at high density at sites that are specialized for the initiation and conduction of action potentials, such as axon initial segments and nodes of Ranvier. In the adult nervous system, the high-density clusters of channels at both initial segments and nodes consist of a specific Na_v channel isoform, Na_v1.6 [1–4], but a different isoform, Na_v1.2, is typically found in unmyelinated axons. This distinction is especially clear in retinal ganglion cells, whose axons are unmyelinated within the eye and then become myelinated after exiting the eye and entering the optic nerve. Na_v1.2 channels are expressed uniformly along the unmyelinated intraocular axons of ganglion cells, but Na_v1.6 channels are sharply restricted to the axon initial segment and nodes of Ranvier [1,2].

During myelination, Na_v1.2 channels accumulate first at immature nodes of Ranvier but are replaced by Na_v1.6 channels as nodes mature, which occurs during the second and third postnatal weeks in the optic nerve [1]. A similar developmental switch from Na_v1.2 to Na_v1.6

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channels also takes place concurrently at the axon initial segments of retinal ganglion cells [2]. Prior to approximately P10, Na_v1.6 immunoreactivity is undetectable in retinal ganglion cells, but by approximately P21, the adult pattern is established. Similarly, Na_v1.6 channel protein levels are low in Western blots of optic nerve extracts prior to about P10, and then rise progressively to adult levels [1]. It is interesting that the developmental increase in Na_v1.6 channel expression parallels the development of repetitive firing in retinal ganglion cells, which make the transition from single spikes to repetitive spikes during sustained depolarization between approximately P14 and P21 [10]. It is not yet clear, however, whether the developmental upregulation of Na_v1.6 channel protein is accompanied by an increase in steady-state levels of Na_v1.6 transcripts. To examine this question, we used real-time, quantitative PCR to determine relative amounts of Na_v1.6 and Na_v1.2 transcripts in mRNA isolated from rat retinas at various postnatal ages spanning the appearance of Na_v1.6 channel protein.

Materials and methods

At each developmental age (P2, P5, P10, P19, P34, and >P60), poly-A RNA was extracted from retinas pooled from litters of rats using the Micro-Poly-A-Pure kit (Ambion), followed by DNase treatment (Ambion, RNase-free). Complementary DNA was synthesized using MultiScribe reverse transcriptase (Applied Biosystems). At each sampled age, four batches of mRNA were prepared from different litters and analyzed separately. All PCR analyses were performed in a total volume of 50 µl using the SYBR-Green PCR Master Mix and an ABI Prism 7700 Sequence Detector (Applied Biosystems). Primers selected for amplification of Na_v1.6, Na_v1.2, and β-actin were of equal efficiency across the range of template concentration used (1–10 ng cDNA). Amplification of β-actin served as an internal reference for each sample [8]. Reactions were performed in triplicate for each of the four samples of reverse transcribed mRNA at each developmental age, and steady-state expression levels for Na_v1.2 and Na_v1.6 transcripts relative to β-actin were calculated across development using the $2^{-\Delta\Delta C_T}$ method [5]. In each experiment, control reactions were included in which reverse transcriptase was omitted (–RT control) and in which no template cDNA was added to the PCR mix (no DNA control). Forward and reverse PCR primers specific for Na_v1.2 and Na_v1.6 alpha subunits were designed within divergent regions near the 3' end of rat sodium channel mRNA, corresponding to the C-terminus of the protein. As shown in Figure 1, the specificity of the primers for Na_v1.2 and Na_v1.6 was verified by PCR from plasmids incorporating the corresponding regions of rat Na_v1.1, Na_v1.2, Na_v1.3, and Na_v1.6. The primers were: Na_v1.2 forward, 5'-TCCGGTTTCGTCACGCTATC-3'; Na_v1.2 reverse, 5'-TCCAGAGAAGACTGATGTGACACC-3'; Na_v1.6 forward, 5'-CAAGCTGGAGAATGGAGGCA-3'; Na_v1.6 reverse, 5'-TAAGAGGGGAGGGAGGCTGT-3'; β-actin forward, 5'-GAGCGCAAGTACTCTGTGTGGA-3'; β-actin reverse, 5'-TCCACATCTGCTGGAAGGTG-3'.

Results

Figure 2 illustrates the developmental change in steady-state levels of Na_v1.6 and Na_v1.2 transcripts, relative to their levels at P2. Examples of single amplification curves for the three transcripts from an individual experiment at P2 are shown in Fig. 2A. For clarity, single curves are shown, although all reactions were actually carried out in triplicate. A threshold level was set within the range of PCR cycles over which the fluorescence signal doubled for each cycle, and the threshold cycle, C_T, necessary to reach threshold was determined for each reaction. After averaging the C_T values across the three reactions for each amplicon, the shift in average C_T (ΔC_T) relative to β-actin was measured for Na_v1.6 and Na_v1.2. As shown in Fig. 2B, ΔC_T was smaller for both Na_v1.6 and Na_v1.2 at P19 than at P2, which indicates an increase in steady-state levels of mRNA for both channel isoforms during developmental maturation of

the retina. The change in ΔC_T from P2 to P19 (i.e., $\Delta \Delta C_T$; [5]) was then used to estimate the fold-change in Nav1.2 and Nav1.6 transcript levels between these two ages. Note, however, that the method does not allow conclusions regarding the amounts of Nav1.2 and Nav1.6 transcripts relative to each other.

Figure 3 summarizes the developmental time course of the increase in mRNA levels for both Nav1.6 and Nav1.2. At P5 and P10, Nav1.6 levels were the same as at P2, but by P19, Nav1.6 mRNA increased approximately 3-fold to a level that was maintained through adulthood. This rise in Nav1.6 transcripts coincides with the appearance of Nav1.6 protein at axon initial segments and nodes of Ranvier of retinal ganglion cells [1,2], which suggests that the increase in steady-state mRNA level gives rise to the increase in protein. The levels of Nav1.2 transcript also increased significantly between P10 and P19, which might at first seem surprising given that Nav1.6 channels replace Nav1.2 channels at initial segments and nodes of Ranvier during this period. However, Nav1.2 channels continue to be expressed in the unmyelinated axons of ganglion cells and in neurites of the inner plexiform layer throughout development, and so the steady-state level of Nav1.2 mRNA would not necessarily be expected to decline. The fact that Nav1.2 transcripts actually rose could indicate that Nav1.2 channel expression increases in these other neuronal compartments at about the time of eye opening, at P14.

Discussion

Although both Nav1.2 and Nav1.6 transcripts rise in parallel after P10, prior to this period only Nav1.2 channels can be detected with immunocytochemistry or Western blots [1]. This implies that expression of Nav1.2 channels before P10 is controlled by a selective mechanism that regulates Nav1.2 only, whereas the later rise in Nav1.6 may be governed by a nonselective mechanism that upregulates multiple sodium channel isoforms, including Nav1.2. In this regard, it is interesting that Nav1.1 channels have also been reported to appear in the retina after P10 [9], consistent with a generalized mechanism controlling expression of Nav channels. In addition, the developmental period from P10 to P19 coincides with the development of the ability of rat retinal ganglion cells to fire repetitively during sustained depolarization [10], which in turn suggests that enhanced repetitive firing might result from increased expression of Nav channels in ganglion cells. However, the density of sodium current in rat ganglion cells has already reached adult levels by P12 [6,7], and Wang et al. [10] attributed the developmental increase in repetitive firing to enhanced recovery of sodium channels from inactivation, not to an overall increase in sodium current density. Therefore, the appearance of repetitive firing after P10 seems more likely to be due to changes in isoform-specific targeting of Nav channels at important sites like the axon initial segment than to an overall increase in Nav channel expression.

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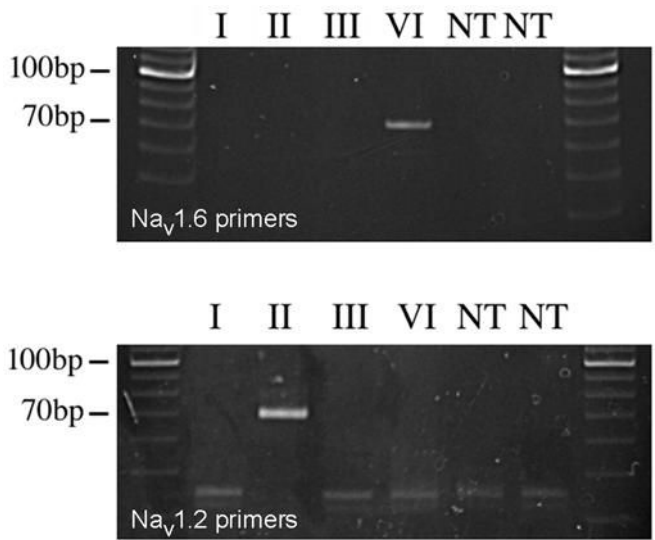


Figure 1. Verification of specificity of primers used for amplification of $Na_v1.6$ and $Na_v1.2$ cDNAs in real-time quantitative RT-PCR. The primers were tested for specificity against plasmids containing the sequence corresponding to the C-terminus of $Na_v1.1$ (I), $Na_v1.2$ (II), $Na_v1.3$ (III), and $Na_v1.6$ (VI) (cloned in PGEM or PGEX-3X vector). Control lanes marked NT represent PCR with no added template, or with undigested template. Products were run on a 10% polyacrylamide gel.

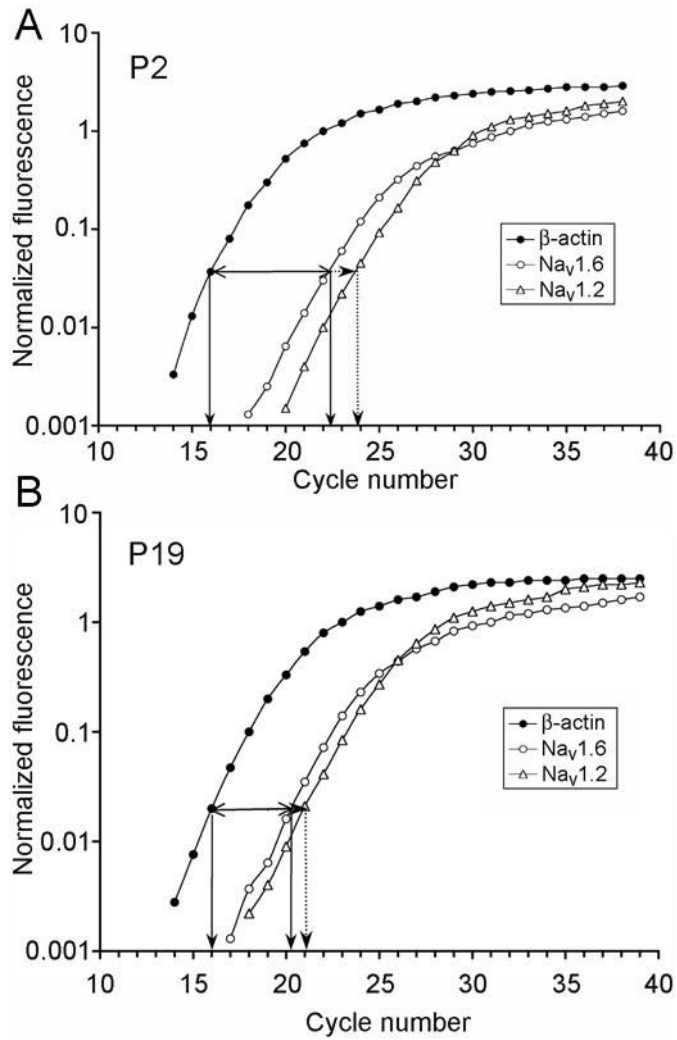


Figure 2. Estimation of amounts of $Na_v1.2$ and $Na_v1.6$ transcripts by quantitative PCR. A. Example fluorescence curves at P2, illustrating the shift in threshold cycle, ΔC_T , for $Na_v1.6$ (solid lines) and $Na_v1.2$ (dashed lines) compared with β -actin. B. Example fluorescence curves at P19, demonstrating the developmental change in ΔC_T for $Na_v1.2$ and $Na_v1.6$.

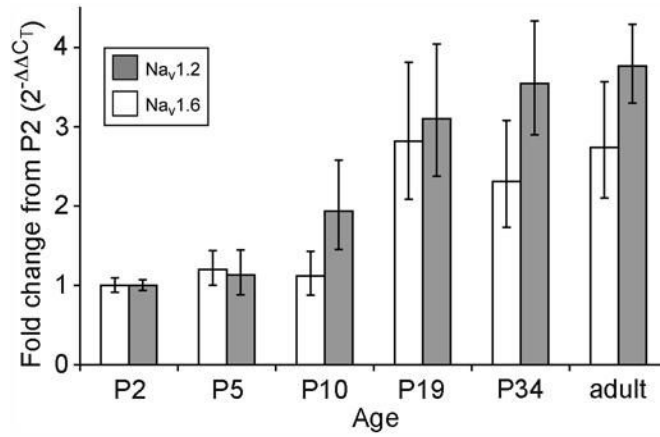


Figure 3.

Developmental increase in steady-state levels of mRNA for Na_v1.2 (white bars) and Na_v1.6 (gray bars), estimated from the shift in ΔC_T relative to P2. Each data point represents the mean of estimates from four RNA preparations. Error bars indicate ± 1 standard deviation. The levels for both Na_v1.2 and Na_v1.6 at P19, P34, and adulthood are significantly elevated compared to P2 ($p < 0.03$).