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TRANSCRIPTIONAL EXPRESSION OF VOLTAGE-GATED NA^+ AND VOLTAGE-INDEPENDENT K^+ CHANNELS IN THE DEVELOPING RAT SUPERFICIAL DORSAL HORN

M. L. BLANKENSHIP, D. E. COYLE AND M. L. BACCEI *

Pain Research Center, Department of Anesthesiology, University of Cincinnati Medical Center, 231 Albert Sabin Way, Cincinnati, OH 45267, USA

Abstract—Neurons within the superficial dorsal horn (SDH) of the rodent spinal cord exhibit distinct firing properties during early life. While this may reflect a unique combination of voltage-gated Na^+ (Na_v) and voltage-independent (i.e. ''leak'') K^+ channels which strongly influence neuronal excitability across the CNS, surprisingly little is known about which genes encoding for Na_v and leak K^+ channels are expressed within developing spinal pain circuits. The goal of the present study was therefore to characterize the transcriptional expression of these channels within the rat SDH at postnatal days (P) 3, 10, 21 or adulthood using quantitative real-time polymerase chain reaction. The results demonstrate that Na_v isoforms are developmentally regulated at the mRNA level in a subtype-specific manner, as $Na_v1.2$ and $Na_v1.3$ decreased significantly from P3 to adulthood, while $Na_v1.1$ was up-regulated during this period. The data also indicate selective, age-dependent changes in the mRNA expression of two-pore domain (K_{2P}) K^+ channels, as TWIK-related acid-sensitive K^+ channels TASK-1 (KCNK3) and TASK-3 (KCNK9) were down-regulated during postnatal development in the absence of any changes in the tandem of pore domains in a weak inward rectifying K^+ channel (TWIK) isoforms examined (KCNK1 and KCNK6). In addition, a developmental shift occurred within the TREK subfamily due to decreased TREK-2 (KCNK10) mRNA within the mature SDH. Meanwhile, G-protein-coupled inward rectifying K^+ channels ($K_{ir}3.1$ and $K_{ir}3.2$) were expressed in the SDH at mature levels from birth. Overall, the results suggest that the transcription of ion channel genes occurs in a highly age-dependent manner within the SDH, raising the possibility that manipulating the expression or function of ion channels which are preferentially expressed within immature nociceptive networks could yield novel approaches to relieving pain in infants and children.

Key words: spinal cord, two-pore-domain K^+ channels, quantitative PCR, neonatal, development, sodium channel.

INTRODUCTION

Mounting evidence suggests that neuronal activity is essential for the proper maturation of nociceptive circuits in the superficial dorsal horn (SDH) of the rodent spinal cord (Beggs et al., 2002). Tactile sensory input from low-threshold primary afferent fibers is an important source of this activity, as it is required for the normal postnatal development of the nociceptive withdrawal reflex (Waldenstrom et al., 2003; Granmo et al., 2008). However, ''pacemaker'' cells which possess the intrinsic ability to generate rhythmic burst-firing have recently been identified within the neonatal SDH, suggesting that the immature spinal cord also contains endogenous sources of neuronal activity which may play a role in the formation of pain pathways (Li and Baccei, 2011), as previously shown for other sensory modalities (Shatz and Stryker, 1988; Tritsch et al., 2007). Many types of pacemaker neurons, including those in the newborn SDH, are distinguished by a high ratio of persistent, voltage-gated Na^+ conductance relative to voltage-independent (i.e. ''leak'') conductance (Del Negro et al., 2002; Li and Baccei, 2011). The prevalence of these burst-firing neurons significantly decreases after the first postnatal week, which is accompanied by an overall decrease in spontaneous activity within the region (Li and Baccei, 2011). Taken together, these findings suggest robust differences in ion channel expression between neonatal and adult SDH neurons.

Nonetheless, little is known about the transcriptional expression of specific ion channel genes within the developing SDH. Recent work has identified $Na_v1.2$ and $Na_v1.3$ as the dominant voltage-gated Na^+ (Na_v) channels in lamina I–II between postnatal days (P) 6 and 30 (Hildebrand et al., 2011), suggesting that the relative expression of Na_v isoforms remains relatively stable during postnatal development. However, since this study did not examine Na_v mRNA levels in the newborn SDH, it is possible that significant changes in Na_v gene expression occur within this region during the

*Corresponding author. Tel: +1-513-558-5037; fax: +1-513-558-0995.

E-mail addresses: blankemh@brandeis.edu (M. L. Blankenship), dennis.coyle@uc.edu (D. E. Coyle), mark.baccei@uc.edu (M. L. Baccei).

Abbreviations: C_t, threshold cycles; K_{2P}, two-pore domain K^+ channels; K_{ir}, inward rectifying potassium channels; $I_{Na,P}$, persistent voltage-gated Na^+ currents; Na_v, voltage-gated Na^+; PBS, phosphate-buffered saline; qPCR, quantitative real-time polymerase chain reaction; SDH, superficial dorsal horn; TALK, TWIK-related alkaline pH-activated K^+ channel; TASK, TWIK-related acid-sensitive K^+ channel; THIK, tandem pore domain halothane-inhibited K^+ channel; TREK, TWIK-related K^+ channel; TRESK, TWIK-related spinal cord K^+ channel; TWIK, tandem of pore domains in a weak inward rectifying K^+ channel.

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first postnatal week. Alternatively, the above developmental alterations in spontaneous activity could potentially reflect the expression profile of two-pore domain (K_{2P}) and inward rectifying (K_{ir}) potassium channels, which are critical determinants of leak membrane conductance and strongly regulate resting membrane potential (V_{rest}) and neuronal excitability across the CNS (Goldstein et al., 2001). Unfortunately, it is currently unclear which subtypes of leak channels are expressed at the mRNA level within the developing SDH.

Therefore, the present study investigated the expression of Na_v, K_{2P} and K_{ir} channels within lamina I–II of the developing SDH using quantitative real-time polymerase chain reaction (qPCR) and immunohistochemical approaches.

EXPERIMENTAL PROCEDURES

Ethical approval

All experiments adhered to animal welfare guidelines established by the University of Cincinnati Institutional Animal Care and Use Committee which approved this study.

Harvesting of SDH

For qPCR experiments, male and female Sprague–Dawley rats were euthanized at postnatal day (P) 3, 10, 21 or 42 ($n = 6$ in each age group). Rats were deeply anesthetized with sodium pentobarbital (30 mg/kg; i.p.) and perfused with ice-cold dissection solution consisting of the following (in mM): 250 sucrose, 2.5 KCl, 25 $NaHCO_3$, 1.0 NaH_2PO_4, 6 $MgCl_2$, 0.5 $CaCl_2$, and 25 glucose continuously bubbled with 95% O_2/5% CO_2. The lumbar (L4-L5) spinal cord was removed and hemisected. The SDH (lamina I–II) was localized via the band of translucence commonly used to identify the substantia gelatinosa and dissected free with a scalpel. Since the translucent band is more difficult to clearly distinguish at P3 compared to later ages, the dissection of the SDH at P3 was based upon previous work which used fluorescent Nissl staining in combination with immunohistochemistry for CGRP and IB4 to measure the thickness of various dorsal horn layers during postnatal development (Lorenzo et al., 2008). Based on these measurements of the thickness of lamina I–II vs. lamina III–VI, the present experiments isolated the top 20–25% of the dorsal gray matter at P3. Any remaining dorsal roots or residual dura mater was then removed and as much white matter was cut away as possible. All steps were carried out in ice-cold oxygenated dissection solution in order to preserve the integrity of the tissue. Following the dissection, residual sucrose solution was briefly rinsed away by immersing the tissue sample in DEPC-treated water. Tissue was then rapidly frozen and stored at −80 °C until use.

RNA isolation

Tissue was homogenized with hand-operated tissue grinders, which were previously treated with RNAse Away (Molecular BioProducts) and baked overnight at 200 °C. Total RNA was isolated with the Norgen BioTek RNA/Protein Purification kit, according to manufacturer's instructions. Briefly, tissue lysate was passed through a nucleic acid binding column, treated with DNAse (Fisher Scientific, Pittsburgh, PA, USA), washed, and eluted in 50 µl of supplied RNA elution solution. The RNA samples were then quantified using Qubit BR-RNA assay (Invitrogen, Carlsbad, CA, USA) with yields ranging from 1 to 3 µg per sample. To normalize RNA concentrations between samples, each sample was precipitated in the presence of 1/10th volume of 3 M sodium acetate and 1 µg of glycogen (as a carrier) in 3 volumes of 100% ethanol for an hour at −80 °C, then reconstituted in DEPC-treated H_2O to yield a final concentration of 0.1 µg/µl. RNA samples were then immediately subjected to reverse transcription (RT) or used for no RT-controls.

Reverse transcription

One microgram of RNA per sample was reverse transcribed with the iScript cDNA kit (Bio-Rad; Hercules CA, USA) containing a mixture of random hexamer and oligo(dT) primers according to manufacturer's instructions, under the following thermal cycler conditions: 5 min at 25 °C, 40 min at 45 °C, 5 min at 85 °C, and held at 4 °C. Each 20 µl reaction was aliquoted to minimize freeze–thaw cycles of the resultant cDNA and stored at −20 °C until use.

Primers

Primers were designed with NCBI/Primer-BLAST tool and BLASTed against the rat Refseq_mRNA database to test for specificity. Whenever possible, primer pairs were designed to contain at least one sequence that crossed an exon–exon boundary to minimize amplification of genomic DNA. Any primer pairs designed without exon boundary restrictions were subject to no-RT control experiments. The specificity of all primers was confirmed using qPCR melting point analysis (MxPro, Stratagene; Santa Clara, CA, USA) and gel electrophoresis. Primers for several candidate reference genes were designed and tested for expression stability using GeNorm and BestKeeper software under the experimental conditions employed in this study, resulting in the choice of HPRT and GAPDH as reference genes. Primer pair sequences can be found in Table 1.

qPCR

Each sample was run in sets of three technical replicates, using 12.5 ng of cDNA, 0.75 µl of 10 µM target-specific primer working solution, 15 µl of prepared SYBR Green qPCR Master Mix (Roche; Indianapolis IN), and nucleic-acid free H_2O to a final volume of 25 µl per reaction. qPCR was performed on a Stratagene MxPro 3500 thermal cycler with an initial 95 °C Taq activation step for 10 min, followed by 40 cycles of the following: 95 °C denaturing step for 15 s, 59 °C annealing step for 30 s, and 72 °C extension step for 30 s, with a fluorescence value recording step (30 s) obtained at 76 °C after every cycle. Following cycling, samples were evaluated for correct product by melting point analysis and considered contamination-free if no-template controls were negative (MxPro, Stratagene).

Data analysis

Raw fluorescence data were normalized to an internal reference dye (ROX) using MxPro software (Stratagene) in order to control for well-to-well pippetting error. These data were imported into LinReg software (Heart Failure Research Center, Amsterdam, The Netherlands) to obtain threshold cycles (C_t), N_0 values, and average plate efficiencies generated from sample amplification kinetics (Ruijter et al., 2009). Replicate outliers were defined as having either a difference of three C_t values from the sample mean or efficiency outside of 5% of the plate mean, and were excluded from further analysis.