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Transcriptional Expression of Voltage-gated Na^+ and Voltage-independent K^+ Channels in the Developing Rat Superficial Dorsal Horn

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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 PCR (qPCR). 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 TASK-1 (KCNK3) and TASK-3 (KCNK9) were down-regulated during postnatal development in the absence of any changes in the 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.

Keywords

spinal cord; two-pore-domain K^+ channels; quantitative PCR; neonatal; development; sodium channel

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

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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 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 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 superficial dorsal horn using quantitative RT-PCR (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 superficial dorsal horn (SDH)

For quantitative PCR 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 superficial dorsal horn (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), 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) with yields ranging from 1 to 3 μg per sample. To normalize RNA concentrations between samples, each sample was precipitated in the presence of $1/10^{th}$ volume of 3M 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) containing a mixture of random hexamer and oligo(dT) primers according to manufacturer's instructions, under the following thermal cycler conditions: 5 minutes at 25 °C, 40 minutes at 45 °C, 5 minutes 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) 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.

Quantitative PCR (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 minutes, followed by 40 cycles of the following: 95 °C denaturing step for 15 seconds, 59 °C annealing step for 30 seconds, and 72 °C extension step for 30 seconds, with a fluorescence value recording step (30 sec) 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) 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.

Data were analyzed according to the "gene expression's C_t difference" formula described previously (Schefe et al., 2006) which incorporates a correction for efficiency in its calculation of fold change in expression. Briefly, the C_t for the target gene at a given age was normalized to a reference gene index (the geometric mean of HPRT and GAPDH expression) from the same sample, then normalized to the same measure in the Adult (i.e. P42) group in order to obtain a "developmental expression ratio" (see Fig 1A). Differences in expression between channel isoforms within the same gene family (see Fig. 1B) were qualitatively assessed by plotting LinReg-derived N_0 values against the mean N_0 value for the reference gene index for all samples. It should be noted that prior studies have compared the relative expression of multiple gene isoforms after accounting for differences in amplification efficiency between primer sets (Donev and Morgan, 2006; Harries et al., 2009). Nonetheless, the comparative analysis of N_0 values does rely on the assumption that the efficiency of the RT reaction within a single tissue sample is the same for all gene isoforms.

Significant effects of age on the developmental expression ratio of a given channel were determined using the Kruskal-Wallis test followed by the Dunn's multiple comparisons test. A value of $p < 0.05$ was considered significant. n refers to the number of animals in a given group. Data are expressed as means ± SEM.

Immunohistochemistry

P21 rats (n = 4–7 for each channel examined) were perfused with freshly prepared 4% paraformaldehyde and the spinal cord lumbar segments removed, then post-fixed overnight at 4 °C. Samples were briefly rinsed in 0.1M PBS (pH 7.4) before being incubated in a gelatin solution containing 20% 75 bloom gelatin and 20% glycerin for 30 minutes, followed by solidification of the gelatin at 4 °C. The gelatin was then cut into blocks, fixed overnight, and transverse sections cut at 50 μm on a vibratome.

Heat-mediated antigen retrieval was performed in the presence of 0.5M citrate buffer for 30 minutes at 85 °C. Sections were washed in 0.1M PBS three times for 5 min each and blocked in 10% normal goat serum in PBS containing 0.3% Triton X-100. Sections were incubated overnight at 4 °C with antibodies raised in rabbit against TASK-1 (1:100; Alomone Labs; Jerusalem, Israel) or TASK-3 (1:50; Santa-Cruz Biotech; Santa Cruz CA) and mouse anti-NeuN (1:500; Millipore; Billerica MA). Control experiments involved pre-treating the primary antibody with a blocking peptide or incubation of the tissue sections with diluted (1:5) blocking buffer in the absence of the primary antibody. Sections were washed in 0.1M PBS containing 0.3% Triton X-100 three times for 30 min and incubated in Alexa 488 goat anti-rabbit IgG and Alexa 594 goat anti-mouse IgG (1:1000) for 2 hours at room temperature. Sections were then washed three times (10 min each), mounted on

Superfrost Plus slides (Fisher Scientific) and coverslipped with Vectashield mounting medium for fluorescence (Vector Labs; Burlingame CA). Sections were imaged on an Olympus BX61 fluorescent microscope equipped with a spinning disc confocal unit under a 40× objective. Images were captured using SlideBook software, and qualitatively assessed for co-localization of NeuN and either TASK-1 or TASK-3 signals in the superficial dorsal horn.

Results

Na_v gene expression within the developing rat superficial dorsal horn (SDH)

To begin identifying which ion channel genes may be important for shaping the firing properties of superficial dorsal horn (SDH) neurons during early postnatal development, we examined the transcriptional expression of voltage-gated Na^+ channel isoforms known to be found in the adult SDH ($Na_v1.1$, 1.2, 1.3 and 1.6; see Fukuoka et al., 2010) using quantitative PCR analysis of RNA isolated from the rat SDH at postnatal days (P) 3, 10, 21 and adulthood (P42). As illustrated in Figure 1A, Na_v 1.1 mRNA was expressed at significantly lower levels during the first two weeks of life, as the developmental expression ratio (see Experimental Procedures) was 4.3 fold lower at P3 compared to adulthood ($n = 6$; $p<0.05$; Kruskal-Wallis test with Dunn's multiple comparison test) and 3.5 fold lower at P10 ($n = 6$; $p<0.05$), before reaching adult levels by P21 ($n = 6$). In contrast, $Na_v1.2$ and $Na_v1.3$ exhibit higher transcriptional expression in the neonatal SDH and are subsequently down-regulated with postnatal age. At P3, the level of $Na_v1.2$ mRNA is 2.8 fold greater than during adulthood ($p<0.01$) and declines to adult levels by P10. Similarly, $Na_v1.3$ expression is 3.9 fold higher at P3 compared to the mature SDH ($n = 6$ for each age group; $p<0.01$ compared to P21 and adult) but declines to adult levels by P10. Interestingly, no age-dependent changes in the relative transcriptional expression of $Na_v1.6$ were observed.

To qualitatively determine the extent to which Na^+ channel gene expression at a particular age is dominated by specific Na_v isoforms, N_0 values for each target gene were normalized to the reference gene index for that age group (see Experimental Procedures). $Na_v1.2$ and $Na_v1.3$ are the most prevalent Na^+ channel transcripts at P3, with minimal expression of $Na_v1.1$ at this age (Fig. 1B). Over the course of development, $Na_v1.2$ and $Na_v1.3$ mRNA levels decline and a more even distribution of the various Na^+ channel isoforms is apparent by adulthood.

Isoform-specific regulation of two-pore-domain K^+ (K_{2P}) channel mRNA within developing spinal nociceptive circuits

Leak potassium currents have been identified as an important determining factor for the level of membrane excitability in the CNS, and K_{2P} channels have been recognized as major sources of this leak current (Goldstein et al., 2001). TASK (TWIK-relxated acid-sensitive K^+ channel) channels represent a subfamily within the K_{2P} group whose members TASK-1 (or $K_{2P}3.1$; corresponding to the KCNK3 gene) and TASK-3 ($K_{2P}9.1$; KCNK9) are known to be expressed within the adult spinal cord (Talley et al., 2000; Berg et al., 2004). We examined the transcriptional expression of these isoforms within the developing SDH using the qPCR approach described above. TASK-1 mRNA levels were ~2.2 fold higher at P3 compared to the P21 or adult groups (Fig. 2A; $n = 6$ for each age group; $p<0.05$; Kruskal-Wallis test with Dunn's multiple comparison test). TASK-1 expression declined toward adult levels after the first postnatal week (Fig. 2A). Similarly, TASK-3 expression was 2.2 fold higher at P3 compared to adulthood ($p<0.05$) and had declined by P10. A qualitative comparison of mRNA levels between the two TASK isoforms examined in the present study suggests that TASK-3 is the dominant isoform at all postnatal ages (Fig. 2B).

TASK channels have been found to be expressed in both neurons and glia in other regions of the CNS (Enyedi and Czirjak, 2010), but little is known about their pattern of expression within the SDH. To determine whether TASK channels are expressed at the protein level within SDH neurons, we used immunohistochemical approaches to examine TASK-1 or TASK-3 expression in the SDH at P21 and evaluated the extent to which these channels co-localized with the neuronal marker NeuN (Fig. 3). TASK-1 immunoreactivity was widespread in the dorsal horn (n = 7), and the majority of labeled cells correspond to neurons as evidenced by their co-expression of NeuN (Fig. 3, *top panels*). Nonetheless, we did observe TASK-1-positive profiles that clearly lacked NeuN expression, suggesting that glial cells within the SDH can also express this subtype of K_{2P} channel. Omission of the primary antibody (Fig. 3, *bottom panels*) or pre-incubation of the primary antibody with a TASK-1 blocking peptide (data not shown) abolished the immunoreactivity in the SDH. While TASK-3 immunoreactivity was detected in fewer cells within the P21 SDH (n = 4), we also observed TASK-3 expression in both neuronal and non-neuronal cells (Fig. 3, *middle panels*).

The TWIK (Tandom of pore domains in a weak inward rectifying K^+ channel) subfamily of K_{2P} channels has been identified in the CNS (Konu et al., 2001) and implicated in setting the resting membrane potential via its contribution to the leak potassium conductance (Lesage and Lazdunski, 2000). However, little is known about the transcription of TWIK channel genes within the SDH or whether their expression is regulated by postnatal age. As illustrated in Fig. 4A, we did not observe an overall age-dependent trend in the expression of the TWIK-1 (K_{2P}1.1; KCNK1) and TWIK-2 (K_{2P}6.1; KCNK6) isoforms, although TWIK-1 mRNA levels were significantly higher at P21 compared to P10 (n = 6 for each age group; $p<0.05$; Kruskal-Wallis test). A qualitative comparison of mRNA levels between the two TWIK isoforms demonstrates that TWIK-1 is the dominant isoform at all ages (Fig. 4B).

The TREK (TWIK-related K^+ channel) subtypes of K_{2P} channels are marked by outward rectification and also contribute to the resting membrane potential (Enyedi and Czirjak, 2010) within the brain and spinal cord (Sano et al., 2003). However, the degree to which the TREK-1, TREK-2 and TRAAK isoforms are expressed at the mRNA level within the developing SDH is unknown. The levels of TREK-1 (K_{2P}2.1; KCNK2) transcripts were independent of postnatal age (n = 6 for each age group; $p>0.05$; Kruskal-Wallis test; Fig. 5A), while TRAAK (K_{2P}4.1; KCNK4) expression was slightly up-regulated during the first three weeks of life with 2.2 fold lower levels at P3 compared to P21 ($p<0.05$). In contrast, TREK-2 (K_{2P}10.1; KCNK10) expression significantly decreased with age, as the neonatal SDH exhibited a 4.3 fold higher level of mRNA compared to the adult ($p<0.01$) before declining by P10. A qualitative comparison of mRNA levels between the three TREK-related isoforms revealed that TREK-2 dominates during early life, but TRAAK appears to exhibit the highest level of expression by adulthood (Fig. 5B). The TREK-1 gene was minimally expressed in comparison to TRAAK and TREK-2 at all ages examined.

The inwardly rectifying potassium channels K_{ir}3.1 and K_{ir}3.2 are also thought to modulate the firing properties of mature spinal neurons (Derjean et al., 2003) and are abundantly expressed within lamina I and II during adulthood (Marker et al., 2006). qPCR analysis demonstrated that the expression of K_{ir}3.1 and K_{ir}3.2 genes remains stable throughout postnatal development (n = 6 for each age group; Fig. 6A). In addition, comparing the mRNA levels of K_{ir}3.1 and K_{ir}3.2 suggests that they are expressed at a similar level throughout life (Fig. 6B).

Discussion

These results show that voltage-gated Na^+ channels (Na_v) and voltage-independent (i.e. "leak") potassium channels are developmentally regulated at the transcriptional level in a subtype-dependent manner within the superficial dorsal horn (SDH) of the rat spinal cord. This suggests that the combinatory expression of ion channel genes which govern intrinsic neuronal excitability within the spinal pain network may be highly dependent upon postnatal age.

Age-dependent modulation of Na_v gene expression within the developing SDH

The present qPCR analysis identifies $Na_v1.2$ as the dominant Na_v isoform in lamina I–II of the spinal cord throughout the first three postnatal weeks, while $Na_v1.1$ mRNA was expressed at low levels during this period (Fig. 1B). These findings are in general agreement with recent work demonstrating that $Na_v1.2$ and $Na_v1.3$ exhibit the highest levels of expression within the rat SDH (Hildebrand et al., 2011). Interestingly, while Hildenbrand *et al.* found that $Na_v1.2$ and $Na_v1.3$ expression did not change between P6 and P30, our data suggest a significant transcriptional down-regulation of both isoforms does occur in the SDH when an earlier period of postnatal development is considered. This supports previous observations that the spinal levels of $Na_v1.3$ mRNA are maximal near birth and significantly decrease by adulthood (Beckh et al., 1989; Felts et al., 1997). Our results also clearly indicate that Na_v genes are regulated independently during the maturation of the dorsal horn network, as $Na_v1.1$ mRNA levels are elevated between the second and third weeks of life in the absence of any changes in $Na_v1.6$ expression (Fig. 1A).

Persistent, voltage-gated Na^+ currents ($I_{Na,P}$) are found in SDH neurons throughout the early postnatal period and may be important determinants of the level of spontaneous activity within developing nociceptive circuits (Prescott and De Koninck, 2005; Li and Baccei, 2011). However, it currently remains unclear as to which of the above Na_v isoforms underlie $I_{Na,P}$ in lamina I–II neurons at various stages of postnatal development. While $Na_v1.1$ (Kalume et al., 2007), $Na_v1.2$ (Rush et al., 2005), $Na_v1.3$ (Lampert et al., 2006) and $Na_v1.6$ (Raman et al., 1997; Smith et al., 1998) are all capable of producing $I_{Na,P}$ in central neurons, these isoforms differ in terms of the fraction of overall Na^+ current which corresponds to $I_{Na,P}$ as well as the effects of β subunits on their inactivation kinetics (Goldin, 1999). Therefore, the age-related shift in the relative expression of Na_v isoforms (Fig. 1) at the mRNA level would be expected to result in developmental changes in the density or voltage-dependent properties of $I_{Na,P}$ within SDH neurons. In the future, it will be important to functionally characterize $I_{Na,P}$ in these cells at various postnatal time points using *in vitro* electrophysiological approaches.

The amplitude of voltage-gated Na^+ currents (I_{Na}) in lamina I–II neurons is markedly up-regulated during the postnatal period, which likely reflects an increased expression of Na_v channels in the axonal, but not somatic, membrane (Safronov et al., 1999). Meanwhile, we observed an overall trend towards decreased Na_v mRNA levels within the mature SDH (Fig. 1B). Collectively, this suggests the possibility that age-dependent changes in post-transcriptional processes underlie the developmental increase in I_{Na}. Another potential contributing factor is the enhancement of Na^+ channel β1 subunit expression which occurs in the rodent dorsal horn during the first 2–3 weeks of life (Sashihara et al., 1995), as β subunits are known to facilitate the delivery of many Na_v subtypes to the cell surface and can also significantly influence the kinetics and voltage-dependence of their gating (Patino and Isom, 2010).

Identification of “leak” channels expressed in the SDH during postnatal development

“Leak” currents refer to background currents present at rest which pass ions in a time- and voltage-independent manner (Goldstein et al., 2001). Potassium-sensitive leak currents strongly modulate neuronal excitability via their effects on the resting membrane potential (V_m), as high K^+ leak conductance maintains V_m at hyperpolarized levels, while lower K^+ leak conductance favors membrane depolarization and action potential discharge. These background K^+ currents can result from any K^+ -permeable channel open at rest, including the voltage-gated K_v channels (Abbott et al., 2001), inward-rectifying K^+ (K_{ir}) channels (Derjean et al., 2003) or the KCNQ subunits underlying M-type K^+ currents in the CNS (Brown and Adams, 1980; Schroeder et al., 2000). Nonetheless, overwhelming evidence now demonstrates that the K_{2P} family of K^+ channels, distinguished by the presence of two pore-forming domains in each subunit, serves as a major source of leak conductance in both neurons and muscle (Goldstein et al., 2001). K_{2P} channels can be classified into six subfamilies as follows (Enyedi and Czirjak, 2010): TWIK (Tandom of pore domains in a weak inward rectifying K^+ channel); TREK (TWIK-related K^+ channel); TASK (TWIK-related acid-sensitive K^+ channel); TALK (TWIK-related alkaline pH-activated K^+ channel); THIK (Tandem pore domain halothane-inhibited K^+ channel) and TRESK (TWIK-related spinal cord K^+ channel). As their names would suggest, the various subfamilies can be regulated in a distinct manner by diverse modulatory factors such as pH, neurotransmitters, temperature, oxygen levels, anesthetics and polyunsaturated fatty acids (Talley et al., 2003). Therefore, it is clear that the particular combination of K_{2P} channel genes expressed by a given neuron will have a profound impact on its intrinsic membrane excitability and strongly influence how environmental stimuli can shape the firing properties of the cell.

To our knowledge, the present study is the first to investigate the transcriptional expression of K_{2P} channels within developing spinal nociceptive circuits. While the amount of mRNA harvested from lamina I–II of an individual spinal cord prohibits qPCR analysis of every KCNK isoform (15 identified to date) in parallel with the Na_v channels of interest (Fig. 1), we have examined selected members of the TWIK, TREK, and TASK families which have previously documented expression in the CNS. Of these, our results suggest that TASK-3 (KCNK9) is expressed at the highest levels in the newborn SDH (Fig. 2B), while both TASK-1 (KCNK3) and TASK-3 mRNA are significantly down-regulated during postnatal development (Fig. 2A). Immunohistochemical co-localization experiments using the neuronal marker NeuN suggest that TASK channels are found in both neurons and non-neuronal cells in the immature SDH (Fig. 3), which supports prior observations that TASK channels are expressed in CNS glia in a regionally-specific manner (Skatchkov et al., 2006; Kim et al., 2008).

As seen for the TASK channels (Fig. 2), the individual members of the TWIK family sampled in the present study exhibited a striking resemblance to each other in terms of their gene expression pattern during development, as neither TWIK-1 (KCNK1) nor TWIK-2 (KCNK6) mRNA levels differed significantly between the neonatal and adult SDH (Fig. 4A). In contrast, we observed a significant age-dependent shift in the distribution of TREK channel transcripts within lamina I–II of the spinal cord. While KCNK4, also referred to as TRAAK due to its activation by arachidonic acid (Fink et al., 1996), was significantly up-regulated during the first three postnatal weeks, the levels of KCNK10 (i.e. TREK-2) mRNA decreased by ~75% over the same period (Fig. 5A). Although the two channels share many functional properties including mechanosensitivity, activation by polyunsaturated fatty acids and regulation by intracellular pH (Lesage et al., 2000; Kim et al., 2001a; Kim et al., 2001b), there are also important differences in their modulation by second messenger pathways. For example, the activation of protein kinase A (PKA) or protein kinase C (PKC) inhibits TREK-2 (Lesage et al., 2000; Gu et al., 2002) but fails to influence TRAAK activity

(Fink et al., 1998). As a result, the higher ratio of TREK-2:TRAAK expression during the neonatal period could enhance the susceptibility of immature SDH neurons to regulation of their leak membrane conductance by G-protein coupled neurotransmitter receptors such as group I mGluRs (Karim et al., 2001; Hu and Gereau, 2003), bradykinin receptors (Kohno et al., 2008) or CGRP receptors (Sun et al., 2004). Unfortunately, little is currently known about how the intrinsic membrane excitability of SDH neurons is modulated by metabotropic inputs during early life.

The $K_{ir}3.x$ family of inward rectifying K^+ channels is comprised of four members ($K_{ir}3.1$–3.4) which are defined by their sensitivity to modulation by the $G_{\beta\gamma}$ subunit of G-proteins in a membrane-delimited manner (Hibino et al., 2010). These channels can be constitutively active in central neurons (Takigawa and Alzheimer, 2002; Chen et al., 2004; Chen and Johnston, 2005) and thus contribute to the leak membrane conductance at rest. The present study chose to examine the transcriptional expression of $K_{ir}3.1$ (i.e. GIRK1) and $K_{ir}3.2$ (GIRK2) in the developing SDH since these isoforms are known to be highly expressed within lamina I–II of the mature spinal cord where they mediate the analgesic effects of μ- and δ-opioids (Marker et al., 2005; Marker et al., 2006). Our results demonstrate that $K_{ir}3.1$ and $K_{ir}3.2$ transcripts are expressed at mature levels in the SDH from birth (Fig. 6), which supports our previous observation that $GABA_BR$-mediated outward K^+ currents are present in newborn lamina II neurons (Baccei and Fitzgerald, 2004).

Methodological Considerations

While the experimental approach employed here provides the ability to quantitatively measure the relative mRNA expression of many channel subtypes in the same tissue sample, this may not necessarily correlate with protein expression and the present study would not detect developmental differences in post-transcriptional processes which regulate ion channel levels within the membrane. Therefore, it will be critical to examine the functional expression of K_{2P} channels in the developing SDH, although selective antibodies or pharmacological agents are not currently available for every K_{2P} isoform. It should also be noted that the cells sampled in these experiments are not restricted to the neuronal population within the SDH. Furthermore, the SDH is characterized by significant neuronal heterogeneity (Graham et al., 2007) and the ion channels examined here may in fact be selectively localized to specific cell types within the spinal nociceptive circuit, as has been previously reported for GIRK channels (Marker et al., 2006). Therefore, while the present study provides an initial examination of the Na_v and leak channels which are found in the SDH at the mRNA level at various stages of development, it will ultimately be necessary to investigate the expression of these channels in identified subpopulations of SDH neurons using immunohistochemistry or single-cell RT-PCR analysis (Cordero-Erausquin et al., 2004; Park et al., 2011), as emphasized by previous work showing that developmental alterations in the intrinsic excitability of SDH neurons occur in a cell-type dependent manner (Walsh et al., 2009; Li and Baccei, 2011; Li and Baccei, 2012).

Conclusions

By beginning to elucidate which genes encoding voltage-dependent and voltage-independent ion channels are expressed in the SDH during early life, this study lays a foundation for future investigations into the molecular mechanisms governing intrinsic membrane excitability within spinal pain circuits in the neonate. The knowledge gained from such investigations could yield insight into the design of improved pharmacological strategies to regulate the activity of central nociceptive pathways in an age-specific manner, which has the potential to improve the clinical treatment of pain in infants and children.

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Highlights

- Na_v and "leak" K^+ channel mRNAs are developmentally regulated in the dorsal horn
- Transcription of $Na_v1.2$ and 1.3 is downregulated during the first postnatal week
- mRNA expression of TASK channels is highest in the neonatal dorsal horn
- TASK-1 and TASK-3 are expressed in both neurons and glia of the spinal dorsal horn
- Age influences the expression of TREK channel genes in a subtype-selective manner

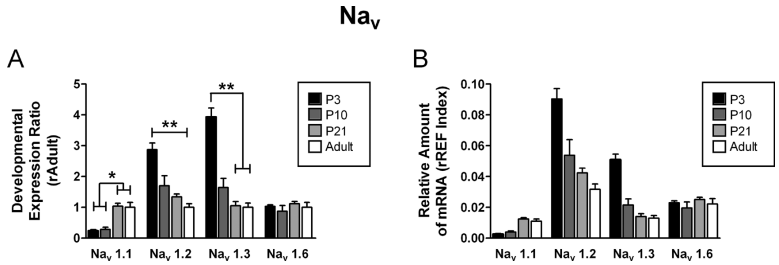

Figure 1. Age-dependent changes in the expression of voltage-gated Na^+ channel (Na_V) genes in the superficial dorsal horn (SDH)

A: Plot of developmental expression ratios (relative to adult; see Methods) for selected voltage-gated Na^+ channel isoforms at P3, P10 and P21 illustrating significant developmental changes in the mRNA levels of $Na_V1.1$, $Na_V1.2$ and $Na_V1.3$ with no alterations in $Na_V1.6$. *p<0.05, **p<0.01; Kruskal-Wallis test with Dunn's Multiple Comparison post-test. *B*: Plot of mRNA amounts relative to a reference gene (rREF) index (see Methods) to allow for the qualitative comparison of relative expression levels between the different Na_V isoforms, demonstrating that Na_V expression at P3 is dominated by $Na_V1.2$ and $Na_V1.3$ with minimal expression of $Na_V1.1$.

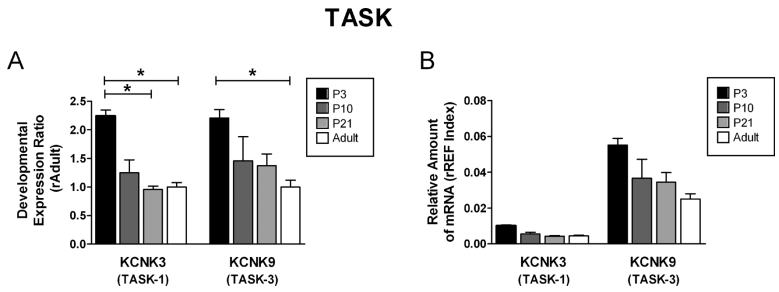

Figure 2. Developmental reduction in TASK K^+ channel mRNA expression in the SDH

A: Both KCNK3 (i.e. TASK-1) and KCNK9 (TASK-3) exhibited a significant decrease in expression over the course of postnatal development. *p<0.05, Kruskal-Wallis test with Dunn's post-test. *B*: KCNK9 was more highly expressed in lamina I–II compared to KCNK3 throughout life.

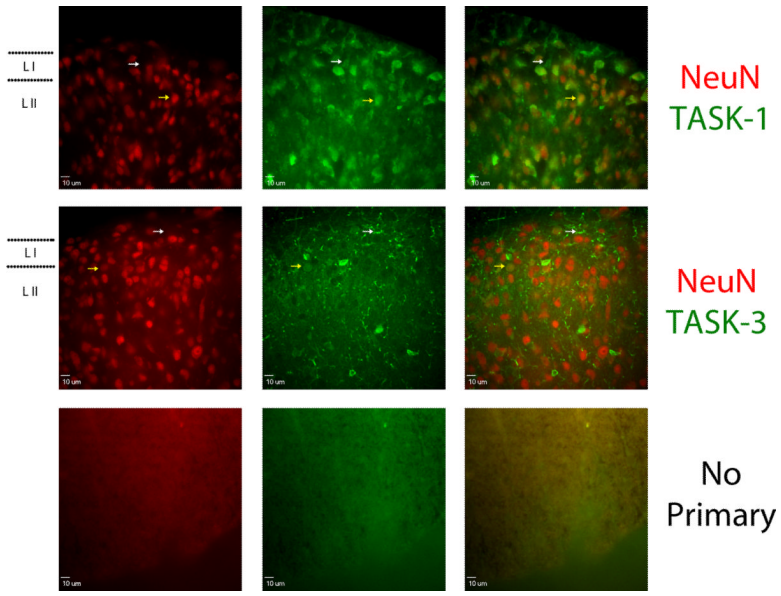

Figure 3. Immunohistochemical localization of TASK channels within the developing SDH

Top: TASK-1 expression (*green*) is co-localized (*yellow arrow*) with neuronal marker NeuN (*red*), and also expressed in NeuN-negative profiles (*white arrow*). *Middle*: NeuN and TASK-3 are co-expressed (*yellow arrow*) and TASK-3 is also expressed in the absence of NeuN (*white arrow*). *Bottom*: Omitting the primary antibody to the TASK channels or NeuN produces no visible signal. The illustrated borders of lamina I–II (*left*) were estimated based on prior work showing that lamina I measures ~40 μm thick throughout postnatal development (Lorenzo et al., 2008).

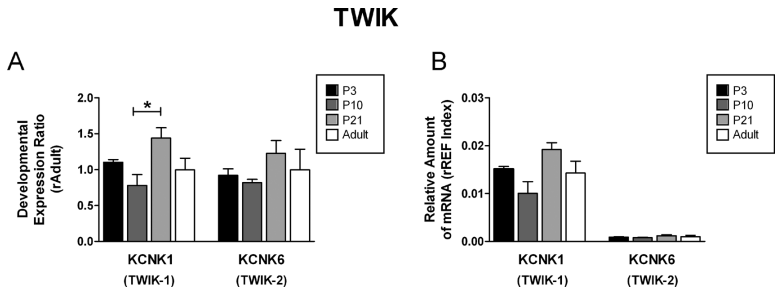

Figure 4. Postnatal expression of TWIK K^+ channel transcripts within spinal nociceptive circuits

A: mRNA levels of KCNK1 (TWIK-1) and KCNK6 (TWIK-2) were not significantly different between P3 and adulthood, though a transient up-regulation of KCNK1 was observed at P21. *p<0.05, Kruskal-Wallis test with Dunn's post-test. *B*: KCNK6 was minimally expressed in lamina I–II throughout the postnatal period.

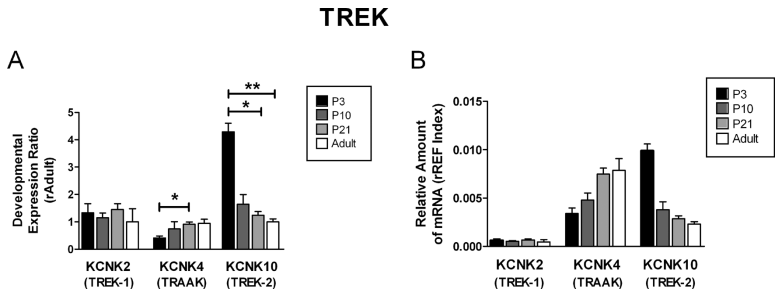

Figure 5. Subtype-specific regulation of TREK K^+ channel mRNA expression within the developing SDH

A: While KCNK4 (TRAAK) mRNA levels were significantly increased during the first three weeks of life, KCNK10 (TREK-2) expression was reduced during the same time period. *p<0.05, **p<0.01; Kruskal-Wallis test with Dunn's post-test. *B*: KCNK4 and KCNK10 are the predominant TREK channels expressed within the SDH throughout development.

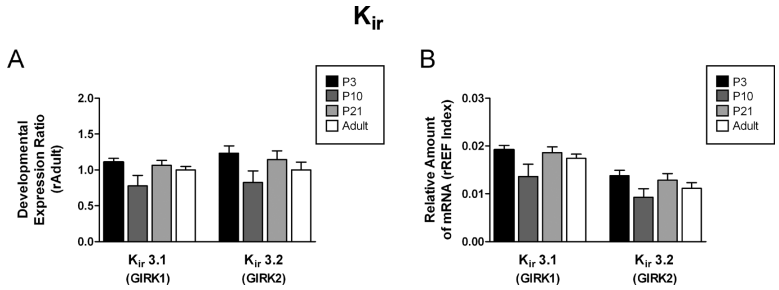

Figure 6. Stable expression of G-protein-coupled inward rectifying K^+ channels (GIRKs) within the developing SDH

A: Neither $K_{ir}3.1$ (GIRK1) nor $K_{ir}3.2$ (GIRK2) exhibited significant age-dependent alterations in mRNA levels within lamina I–II of the spinal cord. *B*: The two isoforms are expressed at comparative levels throughout the postnatal period.

Table 1

Primer Name	Primer Sequence (5->3)
HPRT Forward	TGGATACAGGCCAGACTTTGTTGGA
HPRT Reverse	GGCCACAGGACTAGAACGTCTGC
GAPDH Forward	GGCTCTCTGCTCCTCCCTGTTCTAG
GAPDH Reverse	CGTCCGATACGGCCAAATCCGT
Nav1.1 Forward	AGCCAGCTACTGATGACAATGGAAC
Nav1.1 Reverse	TGCCAGGTCAACAAATGGGTCC
Nav1.2 Forward	TCTCCGCACCAGTCTCTCTTGAGC
Nav1.2 Reverse	TGGCTGACATTGCTAGGACGCC
Nav1.3 Forward	CCGAGCGATTCGGCTTTTGAAAC
Nav1.3 Reverse	GACATTGTCCTGCATCGGAGCC
Nav1.6 Forward	AACGAGAGCTACCTGGAGAACGGC
Nav1.6 Reverse	AGCGGCTCGTAAGGTCAGCTGGTAT
TASK-1 Forward	ACAATCGGCTATGGTCATGCGGCTC
TASK-1 Reverse	GCTGATGCACGACACGAAACCGA
TASK-3 Forward	ATGCAGATCTGTCCGGGCATCC
TASK-3 Reverse	GCAGAACGTGCGTACCCTGT
TWIK-1 Forward	GCCGTTCTGCTGGGATTCGTCA
TWIK-1 Reverse	GAGCAGGTAACACGTGATCCCG
TWIK-2 Forward	GGTGCTTGTCACAGCATACCTCTTC
TWIK-2 Reverse	ATGCAGATCTGTCCGGGCATCC
TRAAK Forward	TTATGTACCAGGCGATGGCACCGG
TRAAK Reverse	CGCTCGCAACCAGTTGCCGATA
TREK-1 Forward	AAAAGGAGCGTCTACCTGGCGG
TREK-1 Reverse	AGCACGGTGGGTTTTGCGGA
TREK-2 Forward	GACCAAGATCCGGGTCATCTCAAC
TREK-2 Reverse	GATCCAAAACCACACCAGCGGC
Kir3.1 Forward	CCTTGGCTCCATCGTGGACGC
Kir3.1 Reverse	GTGTCTGCCGAGATTTGAGCAGCTT
Kir3.2 Forward	TCCACCACAAGGAAAAGCACAAAGA
Kir3.2 Reverse	ACGTTGCCGTGGTGGACGTT