Neuroscience 169 (2010) 828–842

THE DEVELOPMENT OF PERIPHERAL COLD NEURAL CIRCUITS BASED ON TRPM8 EXPRESSION

Y. TAKASHIMA,[a] L. MA[a,b] AND D. D. McKEMY[a,c]*

[a]*Neuroscience Graduate Program, University of Southern California, Los Angeles, CA 90089, USA*

[b]*Department of Cell and Neurobiology, Zilkha Neurogenetic Institute, University of Southern California, Los Angeles, CA 90089, USA*

[c]*Neurobiology Section, Department of Biological Sciences, University of Southern California, Los Angeles, CA 90089, USA*

Abstract—Afferent nerve fibers of the somatosensory system are a molecularly diverse cell population that detects a varied range of environmental stimuli, converting these external cues ultimately into a sensory percept. Afferents mediating detection of thermal stimuli express a repertoire of temperature sensitive ion channels of the TRP family which endow these nerves with the ability to respond to the breadth of temperatures in the environment. The cold and menthol receptor TRPM8 is responsible for detection of cold and, unlike other thermosensors, detects both innocuous and noxious temperatures. How this single molecule can perform such diverse functions is currently unknown, but expression analyses in adult tissues shows that TRPM8 neurons are a molecularly diverse population and it is likely that this diversity underlies differential functionality. To determine how this phenotype is established, we examined the developmental time course of TRPM8 expression using a mouse transgenic line in which GFP expression is driven by the TRPM8 transcriptional promoter (Trpm8^{GFP}). We find that Trpm8^{GFP} expression begins prior to embryonic day 15.5 (E15.5) after which expression reaches levels observed in adult neurons. By E18.5, central axons of Trpm8^{GFP} neurons reach the spinal cord dorsal horn, but anatomical localization and *in vivo* measurements of neural activity suggest that fully functional cold circuits are not established until after the first postnatal week. Additionally, Trpm8^{GFP} neurons undergo a transition in neurochemical phenotype, ultimately reaching adult expression of markers such TRPV1, CGRP, peripherin, and NF200 by postnatal day 14. Thus, based on immunochemical, anatomical and functional criteria, active cold neural circuits are fully established by the second week postnatal, thereby suggesting that important extrinsic or intrinsic mechanisms are active prior to this developmental stage. © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: temperature, TRPM8, cold, menthol, DRG, development.

Sensory neurons of the dorsal root (DRG) and trigeminal (TG) ganglia convert environmental stimuli of a chemical, mechanical, or thermal nature into electrical activity, generating distinct percepts including touch, temperature and pain (Basbaum et al., 2009). Their remarkable ability to differentiate between the various modalities, as well as intensities, is fundamental for appropriate behavioral responses to environmental changes (McKemy, 2007; Basbaum et al., 2009). However, the detailed cellular and molecular mechanisms of each of these sensations are not completely understood (Julius and Basbaum, 2001). A wide repertoire of sensory transduction molecules that convert external environmental stimuli into neural activity have been recently identified, thereby allowing for the examination of the neurochemical, functional and anatomical properties of neurons to which a stimulus modality can be assigned (Basbaum et al., 2009). For example, ion channels of transient receptor potential (TRP) family are the primary detectors of thermal stimuli (Jordt et al., 2003), with the temperatures considered cool to cold mediated by TRP melastatin 8 (TRPM8) (McKemy et al., 2002; Peier et al., 2002; Daniels and McKemy, 2007). Thus, an understanding of the properties of TRPM8-expressing neurons will provide insights into the means by which the peripheral nervous system detects cold temperatures.

Peripheral sensory neurons in general are specified early in development (Marmigere and Ernfors, 2007), with sensory sub-lineages identified even before neural crest precursors become committed to neuronal or glial fates (Zirlinger et al., 2002). However, functionally such distinctions require evidence for intact neural circuits in which neural activity or behaviors to environmental stimuli are produced. The central projections of DRG neurons terminate in a restricted region of the dorsal horn of the spinal cord. In growth of putative small-diameter afferents into the developing dorsal horn occurs in rodents beginning at E19, with nociceptive neurons predominantly terminating in lamina I and II (Mirnics and Koerber, 1995b; Snider, 1998; Hunt and Mantyh, 2001; Julius and Basbaum, 2001). While the establishment of functional spinal neural circuits developmentally is critical for afferent signaling, expression of sensory transduction molecules such as TRPM8 is also required for generation of functional neural responses. Therefore, we hypothesized that functional TRPM8-dependent cold circuits are established when channel expression is initiated and when TRPM8-afferent central projections are strictly localized at lamina I and IIo.

TRPM8 channels are expressed in functionally distinct subsets of neurons associated with unique neurochemical profiles (Takashima et al., 2007; Dhaka et al., 2008). Even more striking is that many of these cells are only visualized by TRPM8 expression and thus not easily defined molec-

*Correspondence to: D. D. McKemy, 3641 Watt Way, HNB 228, University of Southern California, Los Angeles, CA 90089, USA. Tel: +1-213-821-5724.
E-mail address: mckemy@usc.edu (D. D. McKemy).

Abbreviations: CGRP, calcitonin gene-related peptide; DRG, dorsal root ganglia; eGFP, green fluorescent protein; IR, immunoreactive; NGF, nerve growth factor; Trpm8^{GFP}, trpm8 transcriptional promoter.

0306-4522/10 $ - see front matter © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2010.05.039

ularly (Takashima et al., 2007). In the adult, TRPM8 co-localizes with immunoreactivity to the intermediate filaments NF200 and peripherin, markers of Aδ- and C-fibers, respectively, which provide distinct perceptual cold sensations (Jyvasjarvi and Kniffki, 1987; Simone and Kajander, 1997; Takashima et al., 2007). Additionally, a cohort of TRPM8 neurons expresses nociceptor markers such as the painful heat-gated channel TRPV1 as well as calcitonin gene-related peptide (CGRP) (McNeill et al., 1988; Caterina et al., 1997). However, no co-localization between TRPM8 and IB4, a neuronal marker for non-peptidergic neurons, is observed which suggests that those presumptive non-peptidergic neurons (CGRP-negative) observed are not $IB4^+$ neurons. Nevertheless, this strong heterogeneity may account for the broad range of functions attributed to TRPM8 (Welberg, 2008). However, what is not known is when this heterogeneity is established developmentally. To begin to answer this question we used immunochemistry and mouse genetics to examine the somal expression patterns of TRPM8 neurons in the developing mouse embryo, as well as determined when central projections of these afferents are likely to establish functional connections in the spinal cord dorsal horn. These data suggest that cold neurocircuits have the capacity to respond to cold temperatures very early in development, but do not form functional circuits until late in to the second week of postnatal development.

EXPERIMENTAL PROCEDURES

Immunohistochemistry

All animal work was performed in accordance with protocols approved by the Institutional Animal Care and Use Committees at the University of Southern California following the National Institutes of Health regulations. Embryos were collected from pregnant $Trpm8^{GFP}$ transgenic mice with the plug day designated as embryonic day 0.5 (E0.5). Mouse embryos from different embryonic stages were cut on a cryostat (Microm HM560). Sections (16 μm) were processed for immunochemistry as described (Zhao et al., 2009). Postnatal animals were transcardially perfused with 0.1 M PBS followed with fixative (4% formaldehyde, 0.2% (v/v) saturated picric acid, 0.1 M PBS, pH 7.3, at 4 °C). DRGs from lumbar regions 4, 5 and 6 were collected and corresponding sections of spinal cord were carefully dissected and post-fixed overnight at 4 °C in the same fixative solution. Samples were washed with 0.1 M PBS for 30 min. All tissues were cryo-protected in 30% sucrose in PBS at 4 °C overnight, quickly frozen in OCT, sectioned with a cryostat at 12 μm (DRG) and 20 μm (spinal cord), and mounted on Superfrost Plus slides. Slides were stored at −80 °C.

Frozen slides were dried at 4 °C for 30 min and then at 24 °C for additional 30 min and washed with deionized water for 30 s. Slide-mounted sections were washed three times with PBS, once with PBS plus 0.3% Triton X-100 (PBS-T) for 45 min, and three times with PBS before a blocking step (1 h at room temperature with 20% normal goat serum in PBS). Primary antibodies were diluted in a PBS solution and incubated at 4 °C overnight. Antibodies and dilution: 1:1000 chicken anti-GFP (GFP-1020; Aves Laboratories); 1:500 guinea-pig anti-CGRP (T-5027; Peninsula); 1:500 rabbit anti-NF200 (N-4142; Sigma); 1:500 rabbit anti-peripherin (AB-1530; Millipore); 1:500 rabbit anti-TRPV1 (RA-14113; Neuromics); 1:500 rabbit anti-TrkA (a gift from L. Reichardt, UCSF), 1:500 mouse anti-TrkB (610102; BD Transduction Laboratories), 1:500 rabbit PV (AF-1404; R&D system), 1:1000 rabbit anti-c-fos (SC-253; Santa Cruz Biotechnology).

Sections were washed three times with PBS and incubated for 2 h at room temperature with secondary antibodies conjugated to Alexa-488 or Alexa-568 (Invitrogen) diluted 1:1000 in PBS. Slides were then washed three times with PBS, three times with deionized water, and mounted in Vectorshield mounting medium with DAPI (H-1200; Vector Laboratories, Inc.). Digital images were acquired on an Olympus IX70 microscope. Quantification of overlap between green fluorescent protein (eGFP) expression and that of other neuronal markers was obtained per section and expressed as the percentage of GFP-positive cells that were immunoreactive for the respective markers with the SEM between sections obtained from 5 to 12 animals.

For Fos antibody staining, sections were washed three times with PBS and incubated for 2 h at room temperature with (1:200) biotinylated goat anti-rabbit secondary antibody (PK-6101; Vector Laboratories, Inc.) in PBS-T. Slides were washed three times with PBS and incubated for 1 h at room temperature with 1:350 Cy3-conjugated streptavidin (SA1010; Invitrogen) in PBS-T while protected from light. Sections were washed three times with PBS, three times with deionized water, and mounted in Vectorshield mounting medium with DAPI (H-1200; Vector Laboratories, Inc.). Digital images were acquired as described.

Peripheral stimulation

$Trpm8^{GFP}$ transgenic mice at different ages P3, P10, P14, and P35 (P14 and P35 were first anesthetized with isoflurane (Phoenix Pharmaceutical, Inc.)) were treated with vehicle (70% ethanol) or menthol (1 M; Sigma) applied to the ipsilateral hind paw for 5 s repeated 10 times with 1 min intervals. After 90 min, mice were sacrificed and spinal cords dissected, fixed, sectioned, and stained with c-fos antibody as described above. Number of c-fos nuclei was obtained per field and expressed as the number of Fos-positive cells per optical field with the SEM between fields from multiple sections obtained from five to eight independent animals. All datasets were analyzed using two- or one-way ANOVA analysis followed by Tukey's HSD post hoc analysis.

RESULTS

Expression of TRPM8 in the developing mouse embryo

To examine the time course of TRPM8 expression developmentally we used a thoroughly characterized mouse transgenic line in which enhanced GFP (eGFP) is expressed via the $Trpm8^{GFP}$ (Takashima et al., 2007; Carr et al., 2009; Mandadi et al., 2009). $Trpm8^{GFP}$ is robustly expressed and recapitulates expression of TRPM8 channels in peripheral sensory neurons (Takashima et al., 2007), making it a suitable marker for TRPM8 expression and analysis of TRPM8-expressing neurons (Welberg, 2008). We first examined DRG neurons in the developing mouse embryo to establish the embryonic stage at which TRPM8 expression begins. At embryonic day 14.5 (E14.5), we observed very sparse eGFP expression in DRG neurons from $Trpm8^{GFP}$ mice, prior to which no transgene expression was detectable (Fig. 1A). However, by E16.5 eGFP labeling began to increase followed by more extensive labeling by E18.5 (Fig. 1A, B). At E18.5, 8.1±1.0% of DRG neurons (n=3249 cells) were labeled compared to 6.8±0.6% at P0 (n=2931 cells; Fig. 1B). After birth, $Trpm8^{GFP}$ expression was found in a subset of small-to medium-diameter neurons, similar to that we previously