Localization of Na\textsubscript{v} 1.7 in the normal and injured rodent olfactory system indicates a critical role in olfaction, pheromone sensing and immune function

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Abbreviations: AOB, accessory olfactory bulb; BSA, bovine serum albumin; CIP, congenital insensitivity to pain; DAB, 3,3'-diaminobenzidine; DAPI, 4',6-diamidino-2-phenylindole; EDTA, ethylenediaminetetraacetic acid; GFP, green fluorescent protein; GL, glomerular layer; LP, lamina propria; OB, olfactory bulb; OE, olfactory epithelium; OMP, olfactory marker protein; ONL, olfactory nerve layer; PBS, phosphate-buffered saline; RT, room temperature; TTX, tetrodotoxin

Introduction

Voltage-gated sodium (Na\textsubscript{v}) channels are transmembrane proteins that provide a current pathway for the rapid depolarization of excitable cells. The pore-forming \(\alpha\) subunits are classified into nine subtypes denoted Na\textsubscript{v}1.1 to Na\textsubscript{v}1.9.1,2 In recent years, the tetrodotoxin (TTX)-sensitive sodium channel Na\textsubscript{v}1.7 has emerged as a well-validated analgesic target based on several remarkable human genetic studies. Gain-of-function mutations in the \(\alpha\) subunit of Na\textsubscript{v}1.7 (Na\textsubscript{v}1.7) cause congenital indifference to pain and anosmia. We used immunohistochemical techniques to study Na\textsubscript{v}1.7 localization in the rat olfactory system in order to better understand its role in olfaction. We confirm that Na\textsubscript{v}1.7 is expressed on olfactory sensory axons and report its presence on vomeronasal axons, indicating an important role for Na\textsubscript{v}1.7 in transmission of pheromonal cues. Following neuroepithelial injury, Na\textsubscript{v}1.7 was transiently expressed by cells of monocytic lineage. These findings support an emerging role for Na\textsubscript{v}1.7 in immune function. This sodium channel may provide an important pharmacological target for treatment of inflammatory injury and inflammatory pain syndromes.

Loss-of-function mutations in the pore-forming \(\alpha\) subunit of the voltage-gated sodium channel 1.7 (Na\textsubscript{v}1.7) cause congenital indifference to pain and anosmia. We used immunohistochemical techniques to study Na\textsubscript{v}1.7 localization in the rat olfactory system in order to better understand its role in olfaction. We confirm that Na\textsubscript{v}1.7 is expressed on olfactory sensory axons and report its presence on vomeronasal axons, indicating an important role for Na\textsubscript{v}1.7 in transmission of pheromonal cues. Following neuroepithelial injury, Na\textsubscript{v}1.7 was transiently expressed by cells of monocytic lineage. These findings support an emerging role for Na\textsubscript{v}1.7 in immune function. This sodium channel may provide an important pharmacological target for treatment of inflammatory injury and inflammatory pain syndromes.

Von flank, vibration, joint position sense and temperature perception but fail to identify noxious stimuli as unpleasant or painful. In addition to their inability to detect pain, people with CIP have a complete loss of smell (anosmia).14,15 Similarly, mice containing an olfactory-specific knockout of Na\textsubscript{v}1.7 exhibit an absence of odor-guided behaviors.15 Thus, it is clear that Na\textsubscript{v}1.7 plays a critical role in the detection of both nociceptive and olfactory cues.16

The primary olfactory pathway in mammals comprises the olfactory epithelium (OE), cranial nerve I and the main olfactory bulb (OB). The vomeronasal organ and accessory olfactory bulb (AOB) provide a second level of olfactory detection in certain vertebrates, including rodents. While there is debate as to whether or not the vomeronasal organ detects all pheromone-like cues in mammals, and whether its sensory capacity is restricted to pheromonal sensing, the vomeronasal system is generally agreed to be essential for detecting and processing social, behavioral and reproductive cues.17,18

Primary sensory neurons in the OE detect external olfactory stimuli at their dendritic terminals, which causes transmission of action potentials along their axons. These axons converge into...
raised against a unique epitope, was first examined and confirmed in vitro via staining of stably transfected mammalian cell lines expressing different Na\textsubscript{v} channel subtypes. The antibody was found to recognize Na\textsubscript{v}1.7 but not related Na\textsubscript{v} channel subtypes including Na\textsubscript{v}1.4, Na\textsubscript{v}1.5 and Na\textsubscript{v}1.6 (see Fig. S1).

Na\textsubscript{v}1.7 localizes to axons of primary olfactory sensory neurons and central termination zones. We explored the role of Na\textsubscript{v}1.7 in olfaction by studying its expression pattern along the primary olfactory neuraxis. In the nasal neuroepithelium, we observed prominent (++) staining for Na\textsubscript{v}1.7 in the fila olfactoria of the lamina propria (LP) (Fig. 1A and B). We did not detect staining for Na\textsubscript{v}1.7 on the cell bodies and dendrites of primary olfactory sensory neurons in the rat, consistent with recent observations in mice.\textsuperscript{15,23} These findings indicate a particularly high abundance of Na\textsubscript{v}1.7 along the trajectory of the olfactory nerve, which was confirmed by co-localization of olfactory marker protein (OMP) and Na\textsubscript{v}1.7 staining (Fig. 1C), the former being a marker for olfactory sensory neurons and their projections.

Results

We examined the localization of Na\textsubscript{v}1.7 in rat olfactory tissue via standard immunohistochemical and immunofluorescent staining procedures (for details, see Materials and Methods). Specificity of the commercially available anti-Na\textsubscript{v}1.7 antibody, reportedly axonal bundles (fila olfactoria) within the lamina propria underneath the OE. They then pass through the cribriform plate into the nerve layer of the OB, where each axon ultimately synapses with the dendrites of mitral cells (2\textsuperscript{nd} order neuron) in defined OB glomeruli, the functional units of olfactory coding.\textsuperscript{19,20} An axonal bundle projecting out of the OE is generally composed of about 2,000–3,000 olfactory sensory neuron axons wrapped within a tightly connected channel of olfactory ensheathing cells (OECs).\textsuperscript{21} Because of their exposed location, olfactory sensory neurons are constantly replenished from an endogenous stem cell compartment. Therefore, the primary olfactory pathway continuously repairs itself (reviewed in ref. 22).

The precise role of Na\textsubscript{v}1.7 in olfaction has been unclear, with no descriptions of the presence of this channel in the mammalian olfactory system until very recently.\textsuperscript{15,23} We therefore decided to examine the anatomical localization of Na\textsubscript{v}1.7 in the rat olfactory system to gain more insight into its role in olfaction. Our results support the view that Na\textsubscript{v}1.7 plays an essential role in relaying action potentials initiated by the detection of olfactory cues, including pheromones, between the nasal olfactory neuroepithelium and the main OB, as well as between the vomeronasal organ and the AOB. We also show that within this same system Na\textsubscript{v}1.7 is expressed on infiltrating inflammatory cells and thus may play important roles in monocyte actions at sites of tissue injury.

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when viewed at higher optical resolution using confocal microscopy, Na\textsubscript{v}1.7 staining appeared relatively uniformly distributed throughout the glomeruli (Fig. 2C). We again observed colocalization of Na\textsubscript{v}1.7 and OMP staining, confirming the presence of Na\textsubscript{v}1.7 on axons of primary olfactory sensory neurons. At the caudal pole, we observed Na\textsubscript{v}1.7 staining on vomeronasal axons in the AOB, consistent with that of ONL (AOB; Fig. 2D and E).

Expression of Na\textsubscript{v}1.7 on inflammatory cell infiltrate in the injured mouse olfactory epithelium. Because of the emerging role of Na\textsubscript{v}1.7 in immune function,\textsuperscript{24} we studied expression of
this channel in the olfactory pathway after injury. Methimazole drug treatment or Triton-X lesions (data not shown) were used to induce turnover of olfactory sensory neurons and explore expression of Na\textsubscript{1.7} on inflammatory cell infiltrate (Fig. 3).\textsuperscript{25} Treatment with methimazole causes robust recruitment of macrophages due to drug-induced cell death within the olfactory neuroepithelium. Horizontal basal cells, the neuroepithelial stem cells, remain largely unaffected and regenerate the OE in time (~1–2 mo).\textsuperscript{26,27} We examined Na\textsubscript{1.7} staining along the primary olfactory pathway at 2, 5, 10 and 18 d after methimazole injection.

\begin{figure}[h]
\centering
\includegraphics[width=\linewidth]{figure3}
\caption{Degeneration and regeneration of the olfactory epithelium following chemical ablation. (A and B) Representative images showing intact olfactory epithelium (OE) and lamina propria (LP). Well-organized axonal bundles (nb) within the LP are stained for Na\textsubscript{1.7}. Cell bodies of OMP\textsuperscript{+} neurons (~9–10 layers) were also observed in the stratified OE. (C and D) Two days after methimazole injection, a substantial loss of olfactory sensory neurons was evident by the dramatic reduction in OMP\textsuperscript{+} cell numbers. Remnants of olfactory tissue containing OMP\textsuperscript{+} neurons had detached from the basal cells layers and the LP (C, white two-headed arrow). C\textsubscript{x3cr1}\textsuperscript{gfp/+} cells of monocytic lineage (L) were visible within detached epithelial remnants, some of which appeared to co-express Na\textsubscript{1.7} (D). Additional GFP\textsuperscript{+} cells, many with typical macrophage-like morphology, were present in the LP. (E) At 5 d after injury, no OMP staining was visible within the OE, indicating a near complete ablation of mature (OMP\textsuperscript{+}) olfactory sensory neurons following methimazole treatment. Some residual OMP staining was still present in degenerating axon bundles in the LP (white arrowhead). As anticipated, a clear reduction in staining intensity for Na\textsubscript{1.7} was observed at 2 and 5 d post-injury compared with non-injured controls (D and F vs. B). Note, however, the prominent staining for Na\textsubscript{1.7} on inflammatory cell infiltrate accumulating almost exclusively within the LP at 5 d post-injury (F). (M) Immunohistochemical staining for GFP on adjacent sections revealed that cells of monocytic lineage were present in both the LP and OE. At 10 d after injury, the first OMP\textsuperscript{+} cells began to re-appear in the regenerating OE (G, white arrowhead) and little, if any, Na\textsubscript{1.7} immunoreactivity was observed within olfactory nerve bundles (H). By this stage, most of Na\textsubscript{1.7} cell infiltrate had disappeared and the few remaining cells were primarily observed in close association with blood vessels (black arrow). However, numerous macrophage-like cells were present in OE and LP at this time point (N). By 18 d after injury, epithelial regeneration was now clearly evident by a substantial increase in OMP\textsuperscript{+} cell numbers (I) and the reappearance of Na\textsubscript{1.7} staining in olfactory nerve bundles (J). The number and distribution of C\textsubscript{x3cr1}\textsuperscript{gfp/+} macrophage-like cells had also returned to normal control levels (O).\textsuperscript{33} Quantitative counts of GFP\textsuperscript{+} cells within the olfactory neuroepithelium are shown in (K). Note that the appearance of Na\textsubscript{1.7} cells in the LP precedes the peak of macrophage infiltration into the OE. Scale bars: 50 μm.}
\end{figure}
At 2 d following methimazole treatment, the OE appeared severely damaged and disorganized with some regions completely detached from the more basement membrane (compare Fig. 3A and C). Olfactory sensory neurons were virtually absent from the OE at 5 and 10 d after methimazole treatment, as expected, although OMP+ remnants of their axons were still detected within olfactory nerve fascicles (Fig. 3E and G). Regeneration of the OE became evident by a thickening of the cellular layers with time and the reappearance of OMP+ olfactory sensory neurons at 18 d after methimazole treatment (Fig. 3I). Epithelial thickness averaged ~70% of normal control values at this point in time and the intensity of Na1.7 staining returned to normal (++) levels at 18 d.

We noted a high abundance of Na1.7+ inflammatory cells immediately following injury. Specifically, at 2 d following methimazole treatment, and even more so after 5 d, Na1.7+ expression was evident on smaller cells with a globular morphology in the lamina propria underneath the OE (compare Fig. 3D and F). Significantly fewer Na1.7+ cells were observed at later time points (i.e., ≥10 d) after methimazole treatment, at which stage immune-reactive cells were mostly present in close association with blood vessels within the lamina propria (Fig. 3H). As the vasculature of the lamina propria forms the entry point for circulating leukocytes into the OE, these findings suggest that expression of Na1.7 may be downregulated as monocyte precursor cells enter, differentiate and transmigrate into the neuroepithelium for debris clearance and phagocytosis. Such a hypothesis was supported by the fact that numerous macrophage-like cells were present within the lesioned olfactory tissue from Cx3cr1gfp/+ mice at all time points investigated (Fig. 3L–O), and that the appearance of Na1.7+ cells in the lamina propria preceded the peak of macrophage infiltration into the OE (Fig. 3K). Subsequent double-staining procedures confirmed Na1.7 expression on a subset of green fluorescent protein (GFP)+ cells of monocytic lineage. GFP+ Na1.7+ cells lacked the cytoplasmic protrusions more characteristic of mature macrophages, suggesting they were indeed newly recruited monocytes (Fig. 4).

Discussion

The fact that people with CIP as a result of loss-of-function mutations in Na1.7 also suffer from anosmia indicates that this channel must be present in olfactory tissue and that it must play a pivotal role in olfaction. Electrophysiological studies have revealed the presence of TTX-sensitive Na+ currents in cultured rat olfactory sensory neurons and mouse OE tissue slices. All Na+ subtypes except Na1.5, Na1.8 and Na1.9 are TTX-sensitive, but those responsible for Na+ currents in olfactory receptor neurons were unclear until very recently. It is now known that Na1.7 is the predominant Na+ channel in ORNs and that this channel is essential for action potential conduction in olfactory nerve terminals in OB glomeruli.

We observed little, if any, Na1.7 expression on the apical dendrites of both rat and mouse primary olfactory sensory neurons, which is in line with other recent observations in mice and suggests that this channel does not play a direct role in odorant detection. Rather, the observed distribution of Na1.7 within the rat olfactory system, in combination with the severe olfactory deficit in people with CIP that lack Na1.7, indicates that this channel plays a fundamental role in amplifying and propagating action potentials to the OB following the detection of olfactory cues. This function is similar to the role of Na1.7 in nociceptive neurons, where it is believed to act as a threshold channel that amplifies pain signals transmitted above a certain level.

Publication of two independent reports detailing the role of Na1.7 in olfaction in mice coincided with preparation of this paper. We extended the two previous studies on Na1.7 expression along the mouse olfactory pathway by showing that this channel is also present in the rat AOB. The AOB and the vomeronasal organ are responsible for pheromone sensing in mammals. Pheromonal cues are detected within the vomeronasal organ at the base of the nasal cavity and carried via vomeronasal receptor neurons to the AOB, where this information is processed. An earlier report demonstrated through in situ hybridization techniques that Na1.3 is also expressed in the mouse vomeronasal organ, together with Na1.1 and Na1.2, albeit at extremely low levels. Subsequent patch-clamp electrophysiology studies showed that the voltage-dependent Na+ currents were completely inhibited by 2 μM TTX, confirming the presence of TTX-sensitive Na+ channels in rodent vomeronasal organ neurons. The relatively uniform Na1.7 staining we observed in the AOB demonstrates that Na1.7 is present in terminations of the
vomeronal receptor neurons and most likely plays a critical role in pheromone detection. Given that Na\(_1\,3\) is primarily confined to neuronal cell bodies, our findings indicate that, similar to the main olfactory system, the TTX-sensitive Na\(_1\,7\) channel is very likely responsible for the propagation of action potentials along the vomeronal axonal pathway.

Lastly, we noted prominent expression of Na\(_1\,7\) on the early inflammatory cell infiltrate present within the lamina propria after injury. The OE normally contains both macrophages and dendritic cells\(^{33}\) and after injury these populations actively increase through recruitment of circulating monocytes.\(^{34,35}\) Na\(_1\,7\) cellular infiltrate was transiently observed during the acute phase of macrophage recruitment. The use of \(C\_x\_er^{+/+}\) mice revealed that even though a sharply diminished Na\(_1\,7\) immunoreactivity was observed in the sub-acute phase after injury (day 5 onwards), there was a continued presence of monocyte-derived cells within the tissue, suggesting a down-regulation of this channel. These observations are consistent with a recent report showing downregulation of Na\(_1\,7\) during maturation of dendritic cells.\(^{24,36}\) The role of Na\(_1\,7\) in immune cell function and differentiation remains largely unclear. However, emerging evidence suggests a critical role for Na\(_1\,7\) in the maturation and migration of a subset of dendritic cells as well as the production of pro-inflammatory cytokines.\(^{24}\) These findings warrant further investigation into the role of Na\(_1\,7\) in other cells of the monocytic lineage, since therapies that target Na\(_1\,7\) might be useful immune modulators for the treatment of post-injury inflammation and chronic inflammatory pain, in addition to being effective analgesics.

**Materials and Methods**

**Cell culture and immunofluorescence.** Chinese hamster ovarian (CHO) cells stably transfected with Na\(_1\,4\), Na\(_1\,6\) and Na\(_1\,7\) (Genionics AG) were grown on poly-D-lysine coated glass coverslips in F-12 medium (Gibco) supplemented with 9% fetal bovine serum (Lonza, BW14-502E), 0.9% penicillin/streptomycin (Gibco, 15070-063), and 100 \(\mu\)g/ml hygromycin B (Invitrogen, 10687-010). HEK-293 cells stably transfected with Na\(_1\,5\) (Genionics) were grown in Dulbecco’s modified Eagle medium (Gibco, 12491015), 0.9% penicillin/streptomycin and 100 \(\mu\)g/ml zeocin (Invitrogen, R250-01).

Sub-confluent cells were fixed with 10% paraformaldehyde (Sigma, 158127-100G), permeabilized with 10% Triton-X (Sigma, T8532-100ML), then nonspecific binding was blocked by incubation with 10% goat serum (Invitrogen, PCN500). Cells were incubated for 2 h at room temperature (RT) with a rabbit anti-Na\(_1\,7\) antibody (1:2,000; Millipore, AB5390-200UL) and, after additional washing steps, for 1 h with an AlexaFluor 488 goat anti-rabbit antibody (1:1,000; Invitrogen, A11008). Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (1:2,000; Invitrogen, D21490). Coverslips were mounted in fluorescent mounting media (DAKO, S3023) and observed with fluorescence microscopy. To control nonspecific binding, anti-Na\(_1\,7\) antibodies were blocked for 30 min with a 5-fold excess of its antigen or incubated together with nontransfected HEK cells. AlexaFluor 488 goat anti-rabbit antibody alone showed no nonspecific binding (not shown).

**Rat tissue collection and processing.** Male, 5 mo-old Sprague-Dawley rats (n = 5) were deeply anaesthetized via intraperitoneal injection with sodium pentobarbital (80–100 mg/kg; Virbac), followed by transectional perfusion with 100 ml of heparinized saline (0.9% NaCl) and 300 ml of Zamboni’s fixative. The OE and OB were then extracted by dissection followed by overnight post-fixation at 4°C. Samples were subsequently decalcified via overnight immersion incubation in phosphate-buffered saline (PBS) containing 250 mM EDTA (EDTA), followed by cryoprotection in graded (10 and 30%) sucrose solution. Next, tissue samples were snap-frozen in dry ice-cooled isopentane and stored at -80°C until further processing. Tissue sections were sectioned into 20 \(\mu\)m thick slices on a Leica CM 3050 S cryostat and serial sections were collected on Superfrost+ Plus glass slides (Menzel Glaser), air-dried and again stored at -80°C until used.

**Immunostaining procedures.** Slides were air-dried at RT for 40 min after several washes in PBS (3 x 5 min each) for rehydration. For peroxidase-based immunohistochemistry, the tissue was first incubated with 500 \(\mu\)L/slide of quenching solution (0.6% \(\text{H}_2\text{O}_2\) and 10% methanol in PBS) to block endogenous peroxidase activity within the tissue. Slides were then washed again in PBS and blocked with 2% bovine serum albumin (BSA) and 0.2% Triton-X100 in PBS for 1 h at RT in a humidified chamber. Next, slides were incubated overnight at 4°C with primary rabbit polyclonal anti-Na\(_1\,7\) antibody (1:200; Millipore, AB5390-200UL). The following day, slides were extensively washed (3 x 10 min) in PBS to remove unbound antibody followed by incubation with biotinylated goat anti-rabbit secondary antibody (1:400; Jackson ImmunoResearch Laboratories, 711-066-152) at RT. Slides were then washed again and incubated with 1:400 peroxidase-conjugated avidin-biotin complex (ABC; Vector Laboratories, SP-2002) for 1 h at RT. Slides were washed and stained for 3–5 min at RT using 3,3’-diaminobenzidine (DAB) as a chromogen (Sigma Life Science, D4293). The reaction was stopped via immersion of slides into ddH\(_2\)O. Slides were dehydrated through a graded series of ethanol washes (70–100%), cleared in xylene, and coverslipped with Depex mounting medium (Merck, 13514).

For double immunofluorescent staining procedures, similar procedures were followed, except that incubation with quenching solution was omitted. In brief, tissue sections on slides were washed with PBS and blocked with 2% BSA for 1 h. Sections were then incubated overnight at 4°C with a mixture of rabbit anti-Na\(_1\,7\) antibody (1:200) and goat anti-OMP antibody (1:1,000; Wako, 019-2281). Staining for OMP was used to visualize primary olfactory sensory neurons and their projections in the nasal neuroepithelium. The next day, sections were washed (3 x 10 min) and incubated with Cy3-conjugated donkey anti-rabbit (1:400) (Jackson ImmunoResearch Laboratories, 711-165-162) and biotin-conjugated donkey anti-goat secondary antibodies (1:400; Jackson ImmunoResearch Laboratories, 705-065-003) for 1.5 h at RT. Following this, sections were washed (3 x 10 min) and incubated with Alexa 488 conjugated streptavidin (1:400; Sigma, S11223). Finally sections were washed
Microscopy and image analysis. DAB-stained tissue sections were scanned using an Aperio scanscope XT. The staining pattern and distribution were analyzed using ImageScope software (Aperio). Different levels of staining intensity representing the relative abundance of Nav1.7 were documented as: (1) ‘−’ indicates no staining, defined as not above background levels; (2) ‘+’ indicates light staining that is clearly elevated above background levels; (3) ‘++’ for darkly stained regions with the highest abundance of Nav1.7. Photomicrographs of immunofluorescent stainings were taken with an Olympus BX-51 fluorescence microscope and a Zeiss LSM 710 FCS confocal microscope. Greyscale images were false colored and channels were merged to visualize colocalization using Image J.

Olfactory ablation. Methimazole drug treatment or Triton-X lesions were performed as described in references 25 and 37. In brief, mice received two consecutive intraperitoneal injections of methimazole (Sigma, M8506; 50 mg/kg) on days 0 and 3 followed by perfusion and tissue processing as detailed above 2, 5, 10 or 18 d following the first injection.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Supplemental Material
Supplemental materials may be found here: www.landesbioscience.com/journals/channels/article/19484

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