PAIN®

William D. Willis, Jr, MD, PhD Memorial Lecture: The evolutionary history of nerve growth factor and nociception

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1. Introduction

Rare pathological mutations in a small group of genes in humans can lead to pain insensitivity,[4,19] and such cases have been instrumental in driving drug discovery programs for new analgesics.[75] A less frequently trodden track is the examination of why some animals have evolved specific types of pain insensitivity. In this review, I will focus on insights that the biology of African mole-rats has given us in understanding the molecular basis of naturally occurring pain insensitivity.[27,89,91,114] Many millions of years ago, evolution selected for changes in African mole-rat genes producing specific types of pain insensitivity.[27] It is striking that evolution selected for changes in genes and signal transduction pathways that were subsequently identified as being critical for human nociception, which is a major theme of this short review.

It has long been known that naturally occurring chemical substances, called algogens, evoke painful sensations or avoidance in animals and humans. One of the best characterized algogens is capsaicin, a natural substance and the active ingredient of chili peppers, and the substance that confers the burn of hot peppers.[11,12] There is a huge literature on capsaicin, characterizing its neurotoxic effects in neonatal animals, as well as its use as a substance to produce acute experimental pain in humans.[41,124] Capsaicin causes burning sensations in humans and is thought to induce a similar sensation in most vertebrates, one major exception being all birds, which have evolved insensitivity to capsaicin likely because they are seed carriers for capsicum plants.[49] It is not clear when capsaicin insensitivity evolved in the bird lineage, but it is possible that the ancestors of birds, ie, nonavian dinosaurs, were already insensitive to capsaicin. In 2008, we discovered that capsaicin was not a universal irritant in mammals because our studies on the nociceptive system of the naked mole-rat (*Heterocephalus glaber*) revealed that this unusual subterranean mammal was completely insensitive to capsaicin.[91]

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Further experiments also revealed that the naked mole-rat displays no nerve growth factor (NGF)-induced thermal hyperalgesia,[66,91] a phenomenon that is dependent on the presence of the capsaicin-activated ion channel TRPV1.[17]

Naked mole-rats have fascinated biologists for several decades because this underground-dwelling species native to East Africa has a unique biology. Naked mole-rats are 1 of only 2 eusocial mammals that live in very large colonies (up to 300 individuals) with a single breeding female who is the queen.[42,43] In the late 1970s, Dr Jenny Jarvis from the University of Cape Town was the first to show that these animals could be kept and bred in the laboratory, and today naked mole-rat colonies are maintained in many laboratories worldwide. Naked mole-rats have so many extreme physiological features that we would need an entire review to detail them all. Some of their most striking physiological adaptations include an extraordinarily long maximum life span of at least 36 years,[100] apparent resistance to cancer, and resistance to extreme hypercapnia and hypoxia.[92] In this review, I will examine the questions of how and why naked mole-rats, and more recently other African mole-rat species, lost certain types of pain sensitivity.

2. Nerve growth factor: recent history

The naked mole-rat evolved changes in the NGF signaling that probably underlies pain insensitivity in this species.[89,91] Nerve growth factor is the founding member of the neurotrophin family. In the past 20 years, the link between the biology of NGF and pain has been well established.[35,60,73,95] There are at least 5 major pharmaceutical companies running clinical trials of humanized antibodies designed to sequester NGF for the treatment of pain in conditions as varied as osteoarthritis, lower back pain, and interstitial cystitis.[8,14,28,52,57,106] The first example of an NGF-sequestering drug is tanezumab, a humanized monoclonal antibody that potently binds NGF developed by Rinat/Pfizer.[57] At the time of writing, it was not clear whether such drugs will gain regulatory approval because a very small percentage of treated patients had experienced severe joint damage as a side effect of treatment.[106] Because an NGF signaling axis is undoubtedly important in the etiology of pain, it is important to understand how NGF functions in the context of nociception and above all in the context of inflammatory hyperalgesia. Here, I will briefly review the mechanistic basis of how NGF functions in nociception and chronic pain.

The availability of antibody tools to manipulate the endogenous levels of NGF allowed researchers to address the functional consequences of NGF sequestration. Initially, efforts focused on identifying precisely which neuronal populations depend on NGF for survival and when. It is through such experiments and later genetics that we got to know that NGF is required for the survival of

sympathetic ganglion neurons and a large proportion of embryonic sensory neurons that are destined to become nociceptors.[23,101,102] It seems that both sympathetic and sensory neurons largely lose their absolute dependency on NGF for survival in the postnatal period.[101,102]

In the 1980s and 1990s, developmental biologists asked which neurons required the neurotrophins for survival during development.[59] For example, it was known that all sensory neurons that express TrkA (tropomyosin receptor kinase A, also known as NTRK1), the main receptor of NGF during embryonic development, require NGF to survive, but it was also clear that this population is not phenotypically homogenous.[23,74] Nerve growth factor continues to be synthesized in the peripheral targets into adulthood and it has long been noted that the levels of NGF in the target correlate very well with the density of sympathetic and sensory innervation.[54,59,109] Studies in the 1980s already showed that it is primarily neuropeptide-containing nociceptive sensory neurons in the adults that respond to NGF.[59] Thus the neuropeptide content, primarily substance P (SP) and calcitonin gene-related peptide (CGRP), of sensory neurons innervating tissues high in NGF, such as the skin, was observed to be high compared to tissues low in NGF.[78] Lindsay and Harmar had already demonstrated that NGF directly upregulates the SP content of adult sensory neurons.[69] TrkA receptor expression is a feature of all developing nociceptors in the embryo, but its expression is extinguished in postnatal, small-diameter, nonpeptidergic nociceptors.[83] The high-affinity TrkA receptor, which is the primary NGF-signaling receptor, is coexpressed in neuropeptide-positive nociceptors in adults. In mature animals, the peripheral tissue could be shown to influence the chemical composition of sensory afferents. Thus, in experiments where a cutaneous nerve was rerouted to the NGF-poor skeletal muscle and a muscle nerve was rerouted to NGF-rich skin, the SP content changed to match that characteristic of the new target, eg, muscle nerve innervating skin now had a high SP content.[77,78] What was even more striking was the fact that the central connectivity of muscle afferents that had been redirected to skin now resembled that of normal skin afferents.[62] These results led us to conduct the first serious test of the idea that a neurotrophic factor could regulate synaptic strength in the nervous system. We decided to artificially raise the levels of NGF in the skeletal muscle, in this case the gastrocnemius muscle, by chronically pumping NGF into the muscle. By making extracellular recordings from spinal dorsal horn neurons, we knew that only very few of these neurons receive strong synaptic drive from afferents innervating skeletal muscle. However, after exposure to NGF, skeletal muscle afferents showed a huge increase in their ability to excite dorsal horn neurons and this increase was very large when compared to effects of muscle afferents innervating the contralateral, untreated muscle.[68] This was the very first demonstration that a neurotrophic factor can modulate synaptic strength. Shortly afterwards, an elegant and perhaps more direct proof of this idea came from the laboratory of Moo Ming Poo, which showed that both neurotrophin-3 and brain-derived neurotrophic factor (BDNF) can increase the strength of neuromuscular synapses in vitro, with a surprisingly fast time course in the range of seconds.[71] Together, these studies provided the foundation of a huge and important area of study, namely how neurotrophins regulate synapses and synaptic strength in the nervous system.

3. Nerve growth factor and hyperalgesia: the linchpin theory 1993

Hyperalgesia is defined as an increase in the felt intensity of a noxious stimulus, usually after an injury or an inflammatory process. Secondary hyperalgesia is the area of hypersensitivity surrounding an injured area that is not due to peripheral sensitization of the primary afferent because the afferents that innervate the secondary area cannot be directly sensitized by the injury. This type of hyperalgesia is thought to be due to sensitization of central circuits to afferent input coming from nearby the initial injury site.[65,80,124] During the 1980s, it was becoming increasingly clear that the neurobiological basis of secondary hyperalgesia was to a large extent dependent on a phenomenon termed central sensitization.[20,79,127] Thus, strong activation of nociceptors leads to a rapid and long-lasting plasticity at synapses between primary sensory neurons and dorsal horn neurons, and this long-lasting change in synaptic strength can sustain hyperalgesia. Hyperalgesia is induced after injury or inflammation, but can also be produced after skin application of substances that activate or sensitize nociceptors. A classic example of algogen-induced heat and mechanical hyperalgesia is that after application of capsaicin to the skin.[56] While working on the role of NGF in determining the phenotypic identity of nociceptors in Lorne Mendell's laboratory,[61,99] Amy Ritter and myself noted that rats that had been exposed to daily injections of NGF were behaviorally more sensitive to mechanical and heat stimuli than untreated animals. These observations led us to make a more systematic study of the effects of NGF on nociceptive behaviors in the rat. To our surprise, a single systemic injection of NGF (1 mg/kg body weight) produced profound heat and mechanical hyperalgesia, which lasted for several days. Interestingly, heat and mechanical hyperalgesia seemed to be mechanistically distinct because heat hyperalgesia appeared within minutes, whereas mechanical hyperalgesia first became apparent around 7 hours after the injection, becoming maximal and sustained at 24 hours.[67] The fact that a single molecule, NGF, can set into motion a series of rapid functional changes with all the hallmarks of hyperalgesia normally seen after sterile inflammation raised the obvious question of whether NGF was necessary for inflammatory hyperalgesia. This question was particularly pertinent in light of data published showing that NGF was upregulated in the sciatic nerve after inflammation of the skin.[26] It then became obvious to use blocking antibodies in vivo to show whether an inflammation-dependent rise in NGF was a necessary first step in producing hyperalgesia. After obtaining preliminary data using NGF-blocking antibodies, a new model of inflammatory hyperalgesia was proposed where NGF represents a linchpin molecule that provides the key humoral link between inflammation and the nociceptive sensory neurons that initiate and sustain heat and mechanical hyperalgesia.[63] The key features of this model are shown in **Figure 1**, highlighting the areas of progress that have been made since the discovery that NGF is necessary for inflammatory hyperalgesia. Soon after we reported that NGF could induce hyperalgesia, we as well as other groups tested whether NGF-blocking antibodies could ameliorate or block inflammation-associated heat and mechanical hyperalgesia (**Fig. 1A**). The first 2 reports showed that both the heat and mechanical hyperalgesia that follow a complete Freund's adjuvant (CFA)-induced inflammation could be ameliorated by the administration of NGF-blocking antibodies.[67,128] Later on, trkA-IgG fusion proteins (extracellular domain of TrkA fused to the human Fc portion of the immunoglobulin G) that specifically bind endogenous NGF were also shown to be capable of ameliorating heat and mechanical hyperalgesia associated with a carrageenan-evoked inflammation model in rats.[76] The key finding that blockade of NGF-signaling in inflammatory conditions has a major analgesic effect has now been validated in many models.[47,95] It is important to note that recombinant human NGF has also been administered to humans with almost identical effects to those seen in animals.[60,94,103]
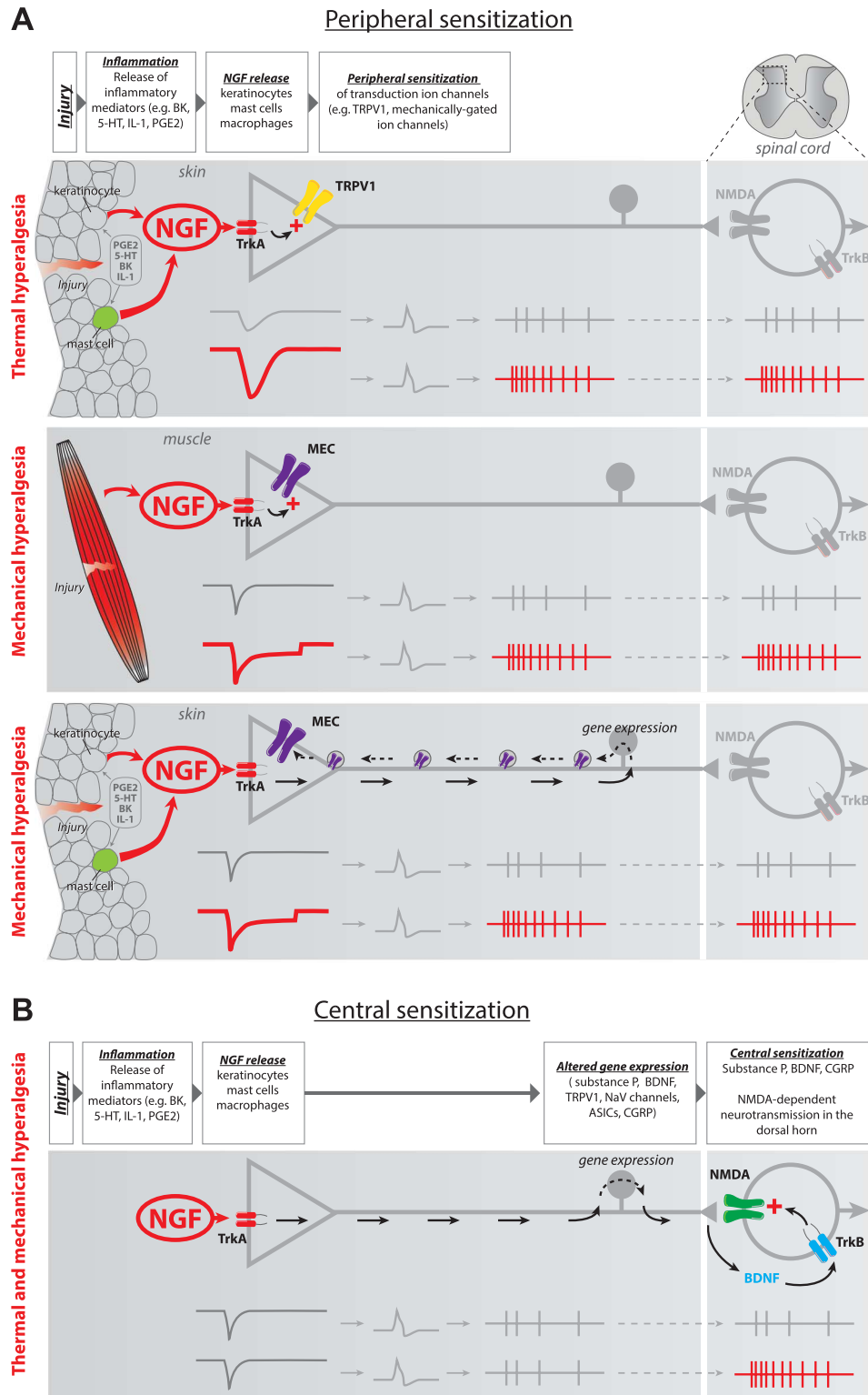
Figure 1. Mechanisms of peripheral and central sensitization. (A) Peripheral sensitization may result from post-translational modifications (top and middle panel) or from increased gene expression and the insertion of additional mechanically gated ion channels in the plasma membrane of the peripheral nerve terminal (bottom). (B) Nerve growth factor (NGF) signaling induces the release of substance P, BDNF, and CGRP from the central terminals of sensory neurons, which sensitize NMDA receptors in second-order projection neurons resulting in the strengthening of synaptic transmission in the spinal dorsal horn—ie, central sensitization.[56] BDNF, brain-derived neurotrophic factor; CGRP, calcitonin gene-related peptide.

We proposed a mechanistic model illustrating the various ways in which increased NGF could produce heat and mechanical hyperalgesia after inflammation.[63] One key feature of this model was the idea that the mechanisms that underlie the NGF-dependent heat hyperalgesia are distinct from those that underlie the mechanical hyperalgesia.[67,67] We supposed that an important difference was that NGF is capable of inducing extremely rapid changes in the peripheral terminals of C-fibers that sensitize

them to noxious heat stimuli. Mechanical hyperalgesia, however, seemed to require the induction of changes in gene expression that eventually leads to central sensitization that maintains mechanical hyperalgesia.[67,67] In the past 20 years, much progress has been made in elucidating the molecular mechanisms that underlie peripheral NGF-dependent heat hyperalgesia. Progress has also been made in understanding NGF-dependent mechanical hyper-algesia and new data indicate that both central and peripheral mechanisms may be important, the molecular basis of which is just beginning to be unraveled, reviewed in 60. One mechanism that seems to be important for the maintenance of central sensitization is the NGF-induced increase in release of BDNF from the central terminals of nociceptors (**Fig. 1B**).[31,34,81] Release of BDNF from the central terminals of TrkA-positive nociceptors activates the BDNF receptor TrkB on spinal interneurons and this is thought to lead to postsynaptic plasticity through phosphorylation of the NMDA receptor (N-methyl-D-aspartate). For a more detailed review on the mechanisms of NGF-induced central sensitization, see Refs. 60, 95.

4. The nociceptive system in naked mole-rats

The first detailed examination of sensory anatomy in the naked mole-rat demonstrated that the skin of this largely hairless species is innervated by both myelinated and unmyelinated fibers.[90] In most rodents, unmyelinated fibers can be subdivided into peptidergic and nonpeptidergic fibers based upon their expression of neuropeptide neurotransmitters such as CGRP and SP.[85,110,118] However, in the naked mole-rat, unmyelinated fibers in the skin were observed to be virtually devoid of SP and CGRP immunostaining. The lack of neuropeptide-positive sensory neurons in the naked mole-rat raised the question of whether this species had lost this sensory population in the course of evolution. One way to examine such a question is to quantify the numbers of unmyelinated axons in peripheral nerves, which is normally done with transmission electron microscopy. Many groups have quantified the numbers of myelinated (A -fibers) and unmyelinated (C-fibers) in cutaneous nerves and consistently observed that there are always 3 to 4 times as many C -fibers as A -fibers in these nerves.[115] However, in naked mole-rats, this ratio is just ~1.5:1 and the low ratio is due to an absolute paucity of unmyelinated nociceptors and not due to increased numbers of A-fiber axons.[89,91,115] Thus, naked mole-rats have far fewer cutaneous nociceptors than other similarly sized mammals, which prompted us to study related subterranean mole-rat species that are phylogenetic neighbors to the naked mole-rat. In a comparative study of nerves from 6 species belonging to the Bathyergidae, the naked mole-rat was the only species with markedly low cutaneous C-fiber to A-fiber ratio.[115] An analysis of the A-fiber and C-fiber numbers related to surface area de-termined that this was accounted for by a paucity of C-fibers, rather than an overabundance of A-fibers.[115] It is likely that mammals have evolved high-density cutaneous nociceptor innervation as the skin is constantly exposed to potentially damaging stimuli. Consistent with this idea, nerves that innervate muscle and joints do not display high ratios of C fibers to A fibers as is seen in the skin and this is true of all mammals examined. Indeed, all bathyergids, including the naked mole-rat, and a wide range of mammals typically display a ~1:1 C-fiber to A-fiber ratio in muscle nerves.[44–46,115] A parsimonious explanation for the cutaneous C-fiber deficit in naked mole-rats is that they largely lack fur, although specialized sensory hairs are present.[22] Compared to most other mammals, humans are relatively naked and lack dense body hair; however, human cutaneous nerves

also show a ~4:1 C-fiber to A-fiber ratio.[87] In normal mice, it has been observed that there is ongoing naturally occurring cell death of sensory neurons with C-fiber axons in the early postnatal period. This loss of axons is reflected in a drop in the C-fiber to A-fiber ratio from ~6:1 in neonates to ~4:1 in adults. We recently observed a similar, but more extreme, process in naked mole-rats, such that postnatal day 3 naked mole-rat pups have a C-fiber to A-fiber ratio of ~6:1 that drops to ~1:1 in adults.[89] The factors that might cause nociceptor loss are discussed below in relation to the biology of NGF and its receptor TrkA.

5. Nociceptor function and nocifensive behaviors in mole-rats

Transgenic mice that lack CGRP and SP show diminished pain behaviors[10,105] and thus the absence of neuropeptides in naked mole-rat nociceptors initially raised the question of whether naked mole-rats display pain insensitivity due to reduced nociceptor function. The existence of nociceptors is almost completely ubiquitous in the animal kingdom and even the nematode worm *Caenorhabditis elegans,* with approximately 300 neurons, has a dedicated set of nociceptors.[113,120] Indeed, the fundamental importance of nociceptors is demonstrated by humans with congenital insensitivity to pain (CIP), a syndrome that can arise due to a complete absence of nociceptive Aδ-fibers and C-fibers.[97,119] The loss of nociceptors observed in some individ-uals with CIP is chiefly due to mutations in NGF or its receptor TrkA.[40] Consistently, mice in which the gene encoding the NGF receptor TrkA was ablated also lack C-fibers.[111] Congenital insensitivity to pain and nociceptor loss is also associated with mutations in other genes, for example, PRDM12 (PRDI-BF1 and RIZ homology domain-containing protein 12).[16] Interestingly, this transcriptional regulator seems to be necessary for normal developmental expression of the NGF receptor TrkA.[25,86]

Despite the fact that cutaneous nerves in naked mole-rats have a depleted number of C-fiber nociceptors, our physiological studies have shown that the receptor properties of these neurons do not differ significantly from C-fibers in mice.[91] This was the case for saphenous nerve Aδ -fibers with nociceptive properties, as well as for C-fiber nociceptors as measured using an ex vivo skin nerve preparation.[82,84,126] The major C-fiber nociceptor population is classified as polymodal receptors (ie, responding both to mechanical and thermal stimuli) and they make up the majority of C -fibers (60%-70%).[30,53,64,65] The second population are C-fibers, which are activated only by intense mechanical stimuli and which make up the remaining functional population seem to have different mechanosensitive properties compared to polymodal C fibers.[6,82] Interestingly, these 2 C-fiber populations were present in naked mole-rats with receptor properties that were almost identical to those of other rodents.[91] Thus, the loss of C-fibers in the naked mole-rat does not seem to be due to loss of one functional subpopulation and the lack of neuropeptides in these C fibers seems to be unrelated to their receptive properties.

The differences in nocifensive behaviors between other rodents and the naked mole-rat were more dramatic when we examined their behavioral response to algogens. Algogens are chemicals that directly produce pain sensation in humans and animals and often work by directly activating specific ion channels or receptors expressed by nociceptors, thereby potently activat-ing sensory receptors that initiate pain. Capsaicin is a prototypical algogen and when injected into mice, evokes a nocifensive licking response, which is due to its specific activation of the transient receptor potential vanilloid 1 channel (TRPV1), a heat-, capsaicin-, and acid-gated ion channel expressed by nociceptors.[12,13,124]

Capsaicin robustly activates naked mole-rat polymodal C fibers as determined by direct electrophysiological recordings with the ex vivo skin-nerve preparation and patch clamp recordings from isolated dorsal root ganglia (DRG) neurons.[89,91,114] However, despite the fact that naked mole-rat nociceptors are activated by capsaicin, it fails to evoke any licking behavior in naked mole-rats.[27,91] In addition, whereas administration of capsaicin causes sensitization of the response to heat (thermal hyperalgesia), this phenomenon also does not occur in naked mole-rats.[91] How then is it that capsaicin activation of nociceptors does not lead to pain behavior? The majority of nociceptive input to the spinal cord terminates in the superficial dorsal horn[121,122] and because DRG neurons are pseudounipolar, proteins that are expressed at the peripheral terminal are also commonly expressed at the pre-synaptic terminal in the dorsal horn. Consequently, one can make recordings from spinal cord neurons and apply capsaicin to activate presynaptic terminals of TRPV1-expressing nociceptors, which produces an excitatory postsynaptic current in the second-order neurons with which TRPV1-positive nociceptors are connected.[129] In mice, the majority of postsynaptic neurons that are synaptically driven by TRPV1-positive nociceptors are located in the superficial dorsal horn, but in the naked mole-rat just as many deep dorsal horn neurons are coupled to capsaicin-sensitive nociceptors as are neurons in superficial layers.[91] This altered connectivity of TRPV1-positive nociceptors potentially explains why capsaicin elicits a robust nocifensive response in mice, but none in the naked mole-rat. Thus, activation of capsaicin-sensitive nociceptors in the naked mole-rat simulta-neously activates both superficial and deep dorsal horn neurons. We speculate that reciprocal connections between synaptically driven interneurons in the superficial and deep dorsal horn could under such circumstances negate a pain signal. Interestingly, when SP was introduced into the naked mole-rat, for example through intrathecal injection, capsaicin injected into the paw was newly able to evoke a nocifensive licking response and provoked thermal hyperalgesia.[91] The neurokinin-1 receptor (NK1R) for SP is only present in the superficial dorsal horn of the naked mole-rat spinal cord and thus presumably activation of nociceptors by capsaicin in the presence of spinal SP leads to a stronger activation of superficial layers that may allow gating of a noci-fensive response.[91] Administration of SP was also shown to "rescue" histamine-induced itch in naked mole-rats[112] and evoke a thermal hyperalgesia that is both NMDA receptor- and NK1R-dependent.[7]

6. Inflammatory pain in mole-rats

In addition to changes in acute nociception, a variety of inflammatory stimuli have been used to examine the development of hyperalgesia in naked mole-rats. Formalin is a substance that evokes acute and late phases of nocifensive behaviors, which although occurring in the naked mole-rat are diminished compared to mice.[27,91] As discussed above, capsaicin fails to evoke thermal hyperalgesia in the naked mole-rat and a similar absence of thermal hyperalgesia was observed after administra-tion of CFA and NGF, although mechanical hyperalgesia was evoked by CFA.[91] Nerve growth factor–induced thermal hyper-algesia is a TRPV1-dependent process,[17] but naked mole-rat sensory neurons respond to capsaicin, which suggests that loss of function in naked mole-rat TRPV1 is unlikely to explain the absence of NGF-induced thermal hyperalgesia. Indeed, sub-sequent analysis of naked mole-rat TRPV1 demonstrated that it has normal heat, pH, voltage, and capsaicin sensitivity.[114] Naked mole-rat sensory neurons also express the NGF receptor TrkA,

but even in cultured DRG neurons, NGF fails to induce sensitization of TRPV1.[89,91] However, NGF sensitizes naked mole-rat TRPV1 when expressed in sensory neurons from mice that lack TRPV1 and when coexpressed with rat TrkA in naked mole-rat fibroblasts, results ruling out deficits in naked mole-rat TRPV1 and the naked mole-rat cellular environment, respectively. Indeed, experiments using naked mole-rat TrkA and a chimeric TrkA (extracellular rat/intracellular naked mole-rat) further showed that activation of the naked mole-rat TrkA receptor is much less efficient at producing NGF-induced TRPV1 sensitization, thus indicating that the deficit lies in the intracellular domain.[89] Furthermore, quantitative proteomics demonstrated that NGF activation of chimeric TrkA receptors is less efficient in engaging downstream signaling as shown by reduced abundance of phosphopeptides compared to rat TrkA. Thus, the lack of NGF-induced thermal hyperalgesia in naked mole-rats is almost certainly primarily due to TrkA hypofunctionality.[89] Examination of the naked mole-rat TrkA sequence shows that between 1 and 3 amino acid substitutions that are unique to the naked mole-rat in the kinase domain of TrkA are likely responsible for the hypofunctional signaling observed.[89] To confirm the role of changes in the TrkA kinase domain underlying the phenotypes observed in the naked mole-rat, one would ideally make a transgenic naked mole-rat with the mouse TrkA sequence, but this is currently not feasible. However, we have generated mice with the critical residues from the naked mole-rat reengineered into the mouse genome. Initial results with these mice indeed indicate that they show reduced inflammatory pain phenotypes (unpublished results). Thus, since the naked mole-rat ancestor appeared more than 30 million years ago, the mole-rat had already blindly discovered the analgesic potential of reduced NGF signaling. The molecular tools available to study laboratory rodents such as mice are extremely powerful in the modern age of deep sequencing and single-cell transcriptomics.[32,125] However, the same methods can also be applied to non-model organisms, such as the naked mole-rat. We recently generated and published a very large data set that enabled us to directly compare the expression levels of nearly 7000 orthologous transcripts from the DRG and spinal cord, not only between the naked mole-rat and mouse, but also across 6 other related African mole-rat species.[27] We have made all these data freely available so that sequence variants across 8 species as well as expression levels of orthologous transcripts can be accessed and analyzed online (https://moleratexpress.mdc-berlin.de). Here, I used these publicly available data to examine the idea that the PRDM12 gene, mutations in which can cause CIP in humans, may show reduced expression or unique sequence variants in the naked mole-rat (Fig. 2). It is clear that 2 CIP genes, Prdm12 and TrkA, show reduced expression in the naked mole-rat DRG compared to the mouse (Fig. 1A); in addition, the Trpv1 also shows reduced expression (Fig. 1A). The PRDM12 gene has been shown to be a key developmental regulator of nociceptor differentiation and is important for the development of TrkA-positive nociceptors.[3,25,86] PRMD12 is a highly conserved protein, which has a polyalanine stretch at the C-terminus, the expansion of which has been directly implicated in pathogenic-ity.[16] Using public databases as well as transcriptomes assem-bled from our own RNAseq data, we aligned the naked mole-rat PRDM12 sequence against human, chimpanzee, mouse, and 2 African rodent species to look for amino acid variants that might be unique (Fig. 2B). Initial analysis indicates that the naked mole-rat sequence has a relatively expanded polyalanine sequence compared to other mammals and possesses 2 seemingly unique amino acid variants in the C-terminal region of the protein. There
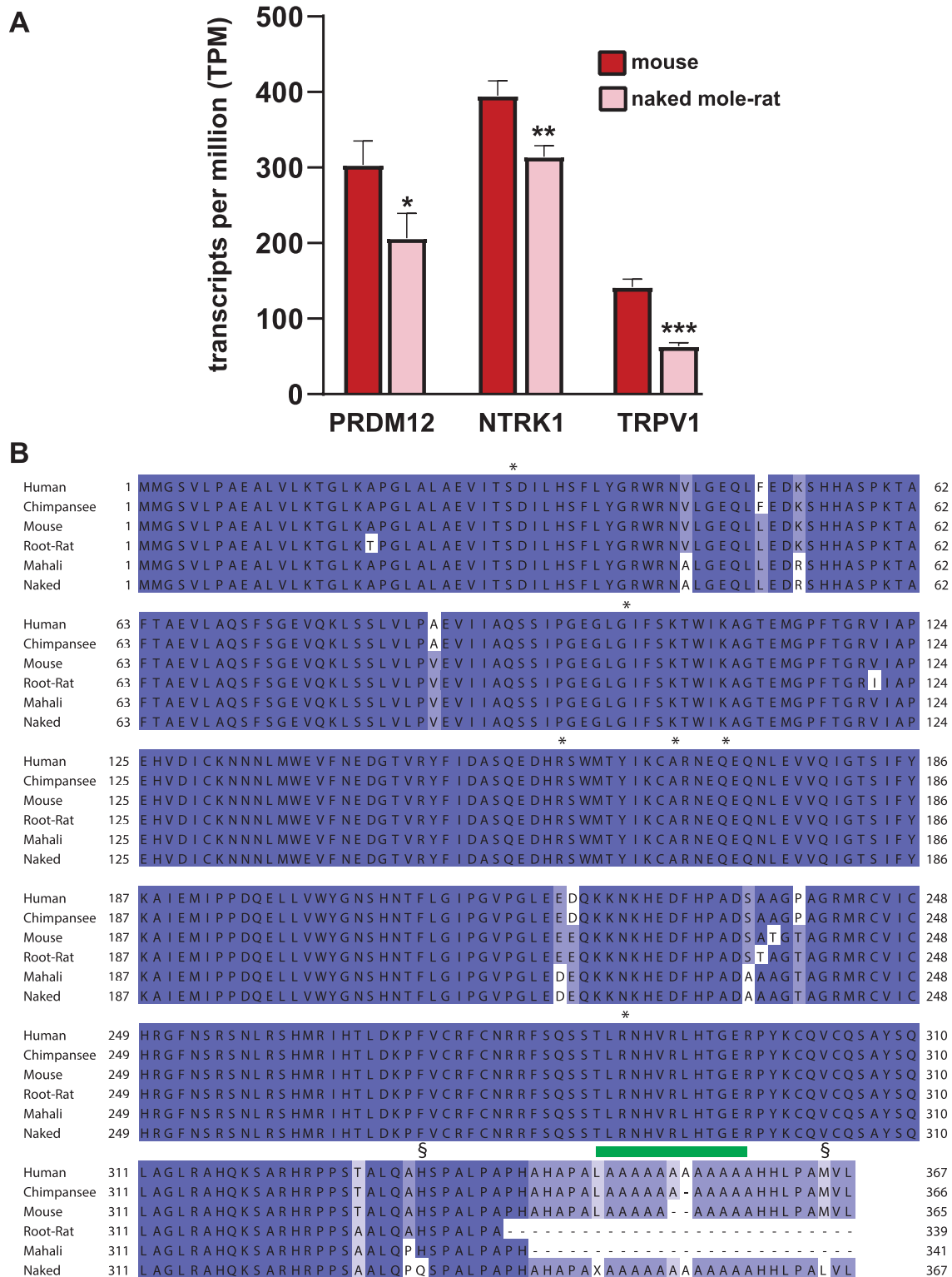
Figure 2. (A) Comparison of mRNA levels for Prdm12, Ntrk1, and Trpv1 in the DRG of mouse and naked mole-rat, using RNAseq data sets downloaded from https://moleratexpress.mdc-berlin.de. All statistics, unpaired t test; PRDM12, $P = 0.0213$; NTRK1, $P = 0.0049$; TRPV1, $P = 0.0003$. (B) A multiple sequence alignment of the predicted amino acid sequence of human mouse and chimpanzee PRDM12 (downloaded from Uniprot) plus alignments with 3 predicted mole-rat amino acid sequences, East African root-rat, and Mahali mole-rat (both sequences down loaded from https://moleratexpress.mdc-berlin.de) and derived from a de novo transcriptome assembly.[25] The naked mole-rat sequence was also downloaded from Uniprot. Note that the root-rat and Mahali sequences may be truncated as an artifact of the de novo assembly. Asterisks (*) indicate positions where human missense mutations were originally identified. Ampersand (§) indicates 2 positions where a unique amino acid variant seems to be present in the naked mole-rat sequence. Green bar indicates the polyalanine sequence that is as long in the mole-rat as it is in the human sequence.

is thus a possibility that this protein may be involved in the development of the unusual nociceptive system in the naked mole-rat. The PRDM12 gene is still expressed in adult sensory neurons (**Fig. 2**), but it is unknown if this protein has a role in maintaining nociceptor physiology in the adult.

7. Absence of acid-induced pain in naked mole-rats

In addition to capsaicin-insensitivity, naked mole-rats also show no pain behavior in response to foot pad injection of acid.[91] Acidic solutions (eg, pH 3.5, similar acidity to lemon juice) cause stinging pain in humans.[98,116] Nocifensive responses to acid solutions have been observed throughout the animal kingdom,[113] which makes the lack of acid response observed in naked mole-rats almost unique (see below). Moreover, although capsaicin activates naked mole-rat C-fibers, no acid excitation of naked mole-rat C-fibers was observed; in mice, all acid-activated C fibers are normally also capsaicin sensitive.[96]

Acid, or more specifically protons, can activate sensory neurons by the modulation or activation of several different classes of ion channels including activation of TRPV1, acid-sensing ion channels (ASICs), and proton-sensing G-protein-coupled receptors and inhibition of certain 2-pore K^+ channels.[38,93] Direct electrophysiological studies have shown that naked mole-rat DRG neurons display depolarizing currents to proton application; indeed, both TRPV1-like and ASIC-like inward currents have been observed[107,114] and expression of different ASIC subunits seems to be roughly equivalent between mouse and naked mole-rat sensory neurons.[108] When examining the proton sensitivity of cloned acid-sensitive proteins, naked mole-rat TRPV1, ASIC1a, and ASIC1b have a similar proton sensitivity to their mouse orthologues[114]; however, the ASIC3 subunit is proton insensitive and seems to shift the proton sensitivity when present in heterotrimers, which may alter sensory neuron acid sensitivity to some extent.[107] It was thus puzzling that isolated naked mole-rat sensory neurons can be excited by protons, but we did not observe acid-driven action potentials in nociceptors nor pain behavior. The first clue to solving this puzzle came with the observation that protons can potently inhibit voltage-gated sodium channel (Na_V) subunits.[51] Voltage-gated sodium channels are necessary for action potential initiation and propagation, as demonstrated by the fact that pathological mutations in some genes encoding Na_V subunits cause CIP.[21,58] Recent work suggests that loss of function mutations in the SCN9A gene encoding $Na_V1.7$ leads to structural changes in nociceptors, which may be the primary reason for nonfunctional nociceptors.[75] The $Na_V1.7$ subunit is important for action potential initiation and setting threshold,[75] and the naked mole-rat gene encodes amino acid variations that when mutated into the human protein considerably enhance proton block of $Na_V1.7$ channels at certain pH values that excite nociceptors.[33,114] We also showed that the low pH is a much more potent inhibitor of mechanically evoked nociceptor firing in naked mole-rat nociceptors than in the mouse. Thus, our model predicts that at least one major mechanism by which naked mole-rats have evolved acid insensitivity is through highly specific changes in residues that enhance proton block of the $Na_V1.7$ channel.[114]

8. Algogen insensitivity: a family affair

The extraordinary sensory attributes of the naked mole-rat recently led us to ask whether pain insensitivity is a commonly occurring attribute among African underground-dwelling rodents. The naked mole-rat belongs to the family Bathyergidae,

a very species-rich group of mole-rats that have spread throughout most of Africa. We systematically tested algogen sensitivity in 8 mole-rat species and one root-rat species from east Africa (*Tachyoryctes splendens*).[27] We used 3 algogen stimuli: capsaicin, acid (pH 3.5 HCl), and allyl isothiocyanate (AITC); the latter molecule is the active pungent ingredient of mustard oil and wasabi and is a highly electrophilic molecule that is a specific agonist of TRPA1 ion channels.[2,36,48,117] So far, not just mammals find AITC and related TRPA1 ligands noxious, but also flies, planaria, reptiles, and birds avoid this substance.[1,50,88,105] We also showed that naked mole-rats display robust nocifensive behavior after hind paw injection of AITC.[27] Thus, naked mole-rats can display the same nocifensive behaviors as all other rodents so far tested.

We discovered that one additional mole-rat species was completely insensitive to capsaicin (the Natal mole-rat *Crytomys hottentotus natalensis*) and 2 additional species were insensitive to acid (Cape mole-rat [*Georychus capensis*] and East African root-rat).[27] The mechanisms that might underlie the loss of acid- and capsaicin-induced pain in African rodents are discussed below. Probably, most surprisingly, we also identified the first case of complete insensitivity to the pungent chemical AITC in the Highveld mole-rat. The Highveld mole-rat is a social African mole-rat that belongs to the *Crytomys* genus and like other members of the genus tested (Common mole-rat, Mahali mole-rat, and Natal mole-rat), all these animals were found to harbor variants in the TRPA1 channel that renders the channel much less sensitive to the AITC ligand.[27] However, the Highveld mole-rat was even impervious to 100% AITC and normally displays no licking or lifting behavior in the first phase of the formalin test.[27] The Highveld mole-rat was also unique among rodents tested so far that it displayed no second phase sensitivity in the formalin test. It is interesting to note in this context that a gain-of-function mutation in the human trpa1 gene is associated with sporadic pain in a large Columbian family.[55] We conducted large-scale transcriptome assemblies from sensory tissues collected from the species tested and quantified changes in gene expression across the phylogenetic tree (**Fig. 3**). This analysis revealed a single upregulated transcript for Nalcn in the Highveld mole-rat that encodes a nonselective leak channel. This voltage-insensitive leak channel (NALCN) has been implicated in carrying a background leak current in neurons.[18,72] We speculated that in Highveld sensory neurons, the hvNALCN protein is overexpressed to shunt excitability of nociceptors that detect AITC. Evidence for this idea was provided by pretreating Highveld mole-rats with an antagonist of the NALCN channel with the hope that this would rescue AITC behavioral sensitivity. We used verapamil, which has been shown to block NALCN channels,[72] and remarkably, after systemic treatment, the Highveld mole-rat became transiently sensitive to both AITC and the effects of formalin.[27] Our data indicate that the Highveld mole-rat has evolved an unusual and effective molecular strategy to become impervious to pain and in principle, such a strategy could be harnessed to develop new analgesics. We also gathered evidence that the Highveld mole-rat lost sensitivity to TRPA1 ligands because it has adapted to sharing its nest with the Natal droptail ant, the venom of which is a potent algogen.[27]

By testing a large number of African mole-rat species for acid sensitivity, we discovered 2 new instances of acid insensitivity, in the Cape mole-rat (*G. capensis*) and the East African root rat (*T. splendens*). The Cape mole-rat and East African root rat are subterranean rodents but are both solitary animals, thus they may not be exposed to the same level of hypercapnic environment probably experienced by the highly social naked mole-rat.
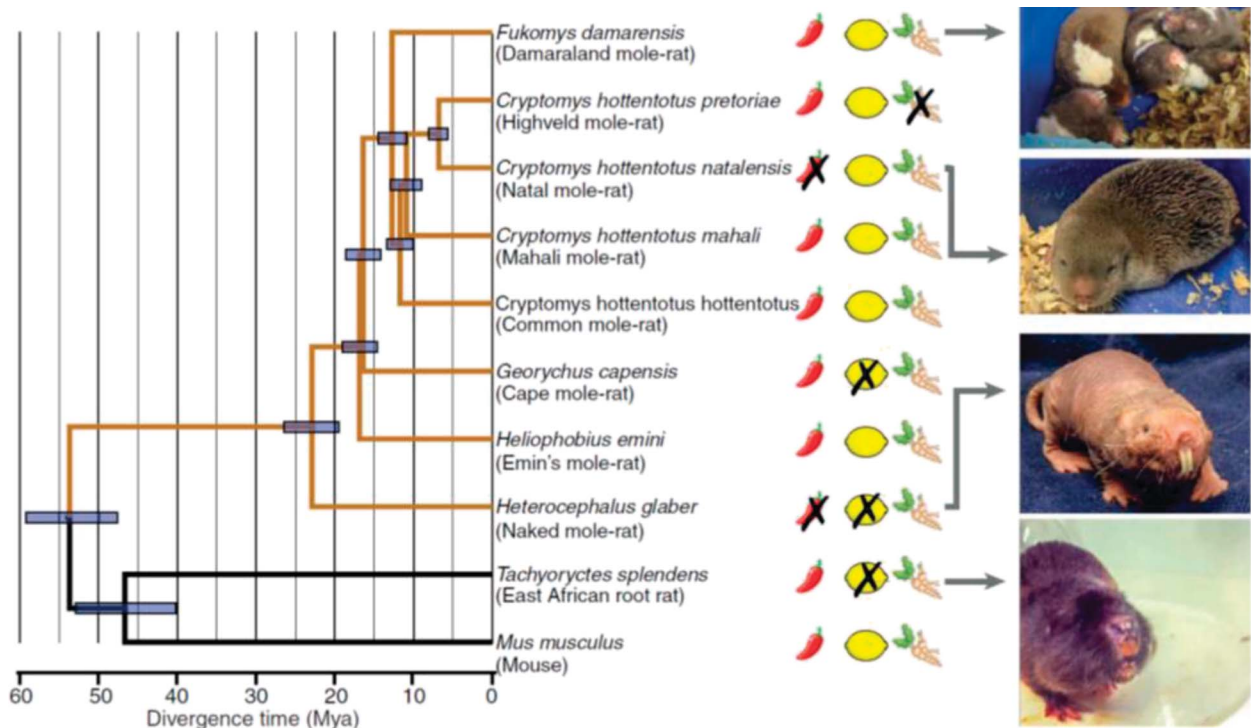
Figure 3. Algogen insensitivities in African rodents. Phylogenetic tree of the studied rodent species, as calculated on the basis of transcriptomic data. Divergence times were calculated on the basis of published and de novo assembled transcriptomes. An "X" indicates an insensitivity to the algogen. Mya, million years ago. (Photo credits [top to bottom]: Karlien Debus, Gary Lewin, Jane Reznick) Illustration courtesy of Science.

Nevertheless, both the East African root rat and the Cape mole-rat have acquired variants in the domain IV of the Na$_V$1.7 channel that convert a normally predominantly positively charged trio of amino acids (KKV, charged $+/+/0$ in mouse and human, single letter amino acid abbreviations) into predominantly negatively charged residues (EKD or EKE, both $-/+/-$ in all subterranean African mole-rats).[24,27,29] It should be noted that EKE variant has been shown experimentally to increase the proton block of the Na$_V$1.7 channel. Interestingly, large-scale sequence analysis of the Na$_V$1.7 channel across the animal kingdom revealed multiple instances of convergent evolution in the same residues of the Na$_V$1.7 channel so that, for example, many hibernating species displayed the naked mole-rat-like EKD variant, but closely related, nonhibernating species exhibited variants resembling the mouse or human sequence.[70] These data provide powerful evidence that the proton sensitivity of the Na$_V$1.7 channel is strongly associated with situations of metabolic stress that could produce tissue acidosis (hibernation is associated with metabolic stress). In our recent study, we found that the acid-insensitive Cape mole-rat was the only species that has the identical amino acid motif in domain IV of the Na$_V$1.7 channel as the naked mole-rat (EKE and not the more common EKD sequence). This is only the second recorded instance of such a motif, which is so far the only motif that has been shown experimentally to increase the proton block of the channel.[27,70] Indeed, detailed analysis also revealed that a functionally equivalent part of the Na$_V$1.7 channel in domain III has amino acid variants that are unique to the acid-insensitive African mole-rats of the family Bathyergidae.[27] The functional importance of such variants, however, remains to be tested. At least in the case of the naked mole-rat, it seems reasonable to assume that exposure to high levels of carbon dioxide as consequence of living in crowded colonies underground could be one factor driving acid insensitivity. Naked mole-rats show an extraordinary resistance to

hypercapnia, which does not produce lung edema in this species.[92] We have also shown that naked mole-rats switch to glycolytic metabolism that produces lactic acid when exposed to extreme hypoxia.[92] It thus seems feasible that acid insensitivity evolved as a protective mechanism to limit pain under metabolically stressful situations. Indeed, in the brain, naked mole-rats seem to have smaller acid-induced currents and decreased acid-induced cell death, suggesting a further protective mechanism.[39] It was recently reported that carbon dioxide levels are elevated in naked mole-rat burrows,[37] but that the level reached is less than that generally associated with hypercapnia-induced changes in respiration rate. However, the levels of carbon dioxide reached in sleeping chambers where the majority of the colony sleep together remain to be measured. We routinely observe that naked mole-rats assemble in piles of sleeping animals to sleep communally every day multiple times, and it is very likely that animals sleeping at the bottom of such piles will be exposed to very low oxygen and high carbon dioxide.

Because the 3 acid-insensitive species observed here seem to occupy different types of habitats, it was all the more surprising that we could also detect multiple common genes that were regulated in the same direction in the DRG of all 3 species. We could show that all 3 species showed reduced levels of transcripts encoding ion channels whose activity would be enhanced by protons.[27] Examples of such genes included TWIK1 and ASIC3.[15,108] However, we also discovered multiple new genes involved in metabolic processes that may also help all 3 species cope with acidosis.

9. Why did selective pain insensitivities evolve?

It is hard to prove that loss of physiological trait in the course of evolution, examples described here being sensitivity to acid,

capsaicin, or AITC, confer a selective advantage to the species in question. In some cases, we can speculate about the benefits that the loss of algogen sensitivity might bring the species, eg, acid insensitivity in the naked mole-rat may help this species deal with tissue acidosis associated with hypercapnia associated with crowded underground living.[92] Nevertheless, it is also possible that some pain-insensitivity traits may have evolved for reasons that are not primarily associated with pain per se. For example, the specific loss of C-fiber nociceptors that was observed only in the most social mole-rat species, the naked mole-rat, may be an adaptation to a need for energetic efficiency rather than an adaptation to it sensory world. The naked mole-rat occupies a very food resource-poor niche, which puts energy efficiency at a premium. Thus, it has been proposed that other adaptive traits in this species, such as lack of fur and lack of thermogenesis, are tradeoffs made to reduce energy expenditure where it is not strictly required.[5,9,43] Similarly, loss of some, but not all C-fibers, that consume energy may also be a trade-off that benefits the general fitness of the species.

In conclusion, studies on African mole-rats have so far revealed novel molecular strategies to ameliorate pain. Strikingly, the molecular changes used by mole-rats to reduce pain have turned out to be remarkably overlapping with the causes of human CIP. Of the 6 main genes associated with CIP in humans, 2 (TrkA and $Na_V1.7$) are directly associated with pain insensitivity in naked mole-rats. Non-model rodents represent a highly valuable resource to discover how evolution can produce novel solutions that will in the end inform us how to best develop therapeutic approaches for a variety of human illnesses as well as pain.

Conflict of interest statement

The author has no conflicts of interest to declare.

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