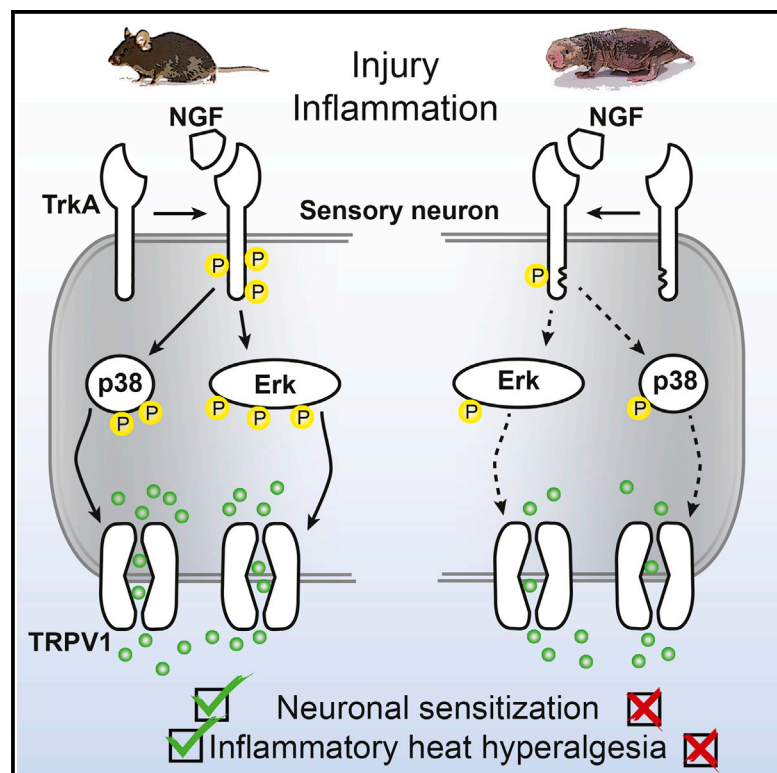


Hypofunctional TrkA Accounts for the Absence of Pain Sensitization in the African Naked Mole-Rat

Graphical Abstract



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In Brief

Omerbašić et al. show that absent pain sensitization in naked mole-rats is associated with hypofunctional TrkA signaling. NGF stimulation of TrkA normally sensitizes TRPV1 channels, but not in naked mole-rat sensory neurons. The naked mole-rat TrkA kinase domain was shown to contain amino acid variants that attenuate TRPV1-dependent pain sensitization.

Highlights

- TRPV1 ion channels in naked mole-rat nociceptors are not sensitized by NGF
- Naked mole-rat TRPV1 channels are sensitized by NGF in mouse nociceptors
- NGF activation of naked mole-rat TrkA receptors does not sensitize TRPV1
- One to three amino acids in the naked mole-rat TrkA receptors may render it hypofunctional



Hypofunctional TrkA Accounts for the Absence of Pain Sensitization in the African Naked Mole-Rat

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SUMMARY

The naked mole-rat is a subterranean rodent lacking several pain behaviors found in humans, rats, and mice. For example, nerve growth factor (NGF), an important mediator of pain sensitization, fails to produce thermal hyperalgesia in naked mole-rats. The sensitization of capsaicin-sensitive TRPV1 ion channels is necessary for NGF-induced hyperalgesia, but naked mole-rats have fully functional TRPV1 channels. We show that exposing isolated naked mole-rat nociceptors to NGF does not sensitize TRPV1. However, the naked mole-rat NGF receptor TrkA displays a reduced ability to engage signal transduction pathways that sensitize TRPV1. Between one- and three-amino-acid substitutions in the kinase domain of the naked mole-rat TrkA are sufficient to render the receptor hypofunctional, and this is associated with the absence of heat hyperalgesia. Our data suggest that evolution has selected for a TrkA variant that abolishes a robust nociceptive behavior in this species but is still compatible with species fitness.

INTRODUCTION

Inflammation and tissue injury cause hypersensitivity of the affected tissue so that mild mechanical and thermal stimuli become painful. This phenomenon is called hyperalgesia (Lewin et al., 2014; Smith and Lewin, 2009). A critical endogenous mediator of inflammatory thermal and mechanical hyperalgesia, both in rodents and in humans, is nerve growth factor (NGF). Early studies in rodents and humans revealed that a single local dose of exogenous recombinant NGF can produce profound and long-lasting thermal and mechanical hyperalgesia (Dyck et al., 1997; Lewin and Mendell, 1993; Lewin et al., 1993; Petty et al., 1994). Furthermore, loss of function mutations in the NGF gene or *NTRK1*, which encodes the high-affinity NGF re-

ceptor TrkA, cause a range of congenital pain insensitivity syndromes in humans (Carvalho et al., 2011; Einarsdottir et al., 2004; Indo et al., 1996). The NGF/TrkA signaling system is critical for the genesis and maintenance of hypersensitivity states in mammals (Lewin et al., 1994, 2014; Woolf et al., 1994). The importance of increased NGF signaling during pain has recently been reinforced by the fact that blocking NGF signaling appears to be highly effective in treating pain in humans on the basis of phase 2 clinical trial data (Katz et al., 2011; Lane et al., 2010).

The naked mole-rat (*Heterocephalus glaber*) is a eusocial African rodent that displays a range of extreme physiological characteristics from cancer resistance and extreme longevity to complete insensitivity to acid (Liang et al., 2010; O'Connor et al., 2002; Park et al., 2008; Smith et al., 2011; Schuhmacher et al., 2015). We discovered that this species completely lacks behavioral heat hyperalgesia when challenged with NGF and the pro-inflammatory agents capsaicin and complete Freund's adjuvant (Park et al., 2008). The polymodal, capsaicin-gated ion channel TRPV1 is also required in mice for the development of NGF-induced heat hyperalgesia (Chuang et al., 2001). However, our studies have shown that although naked mole-rats are behaviorally insensitive to capsaicin, they have sensory neurons that express a TRPV1 channel with ligand sensitivity and biophysical properties indistinguishable from that found in mice or humans (Smith et al., 2011). Here, we investigated how heat hyperalgesia has been disabled in the naked mole-rat over the course of evolution. We addressed this question using molecular and cellular approaches to dissect out at which stage of the sensitization pathway heat sensitization fails. A cellular model of heat hyperalgesia is the rapid and potent sensitization of TRPV1 currents that has been studied in isolated sensory neurons (Shu and Mendell, 2001). We show that rapid sensitization of TRPV1-mediated currents is absent in sensory neurons from naked mole-rats. However, the naked mole-rat TRPV1 protein can be sensitized when expressed in mouse sensory neurons. We show that the cloned naked mole-rat TrkA receptor is less efficient at engaging signal transduction pathways leading to TRPV1 sensitization. Furthermore, we demonstrate that unique amino acid variants in the kinase domain of the naked mole-rat

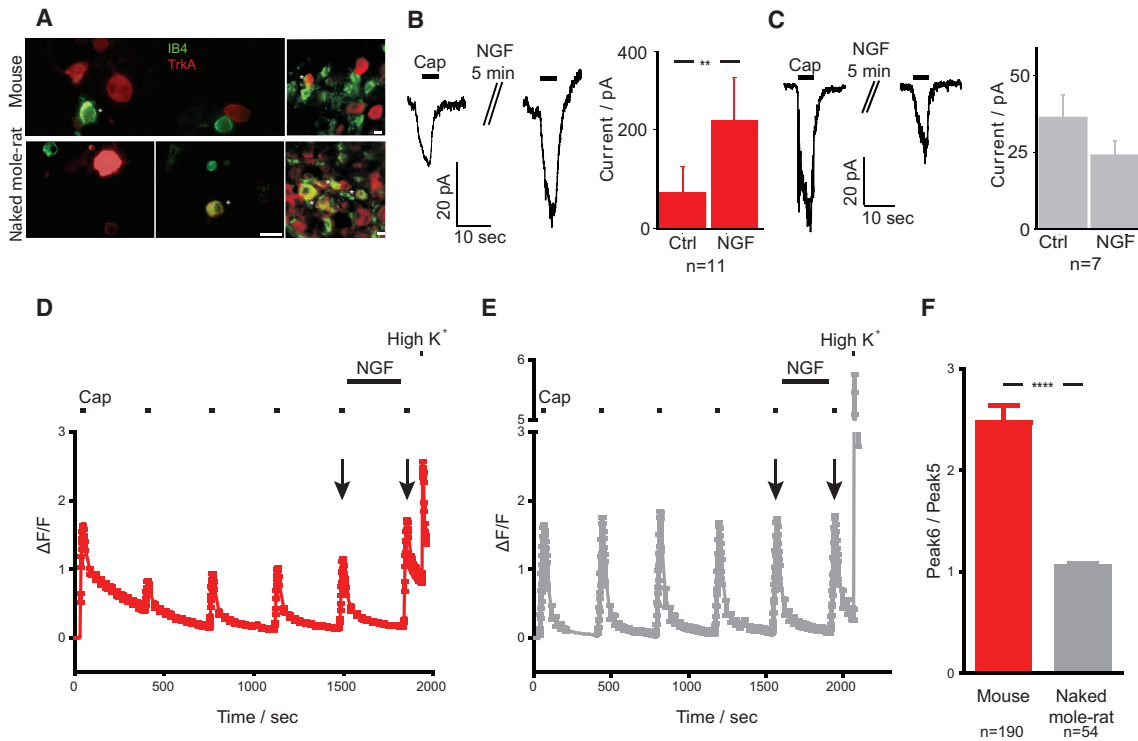


Figure 1. Naked Mole-Rat Dorsal Root Ganglia Neurons Are Not Sensitized by NGF

(A) IB4 (green) and TrkA (red) label largely different populations of mouse DRG neurons, greater co-labeling occurs in naked mole-rat TrkA-positive DRG neurons in culture (left panels) and sections (right panels). Asterisks denote double-labeled neurons; scale bar, 20 μ m. (B and C) NGF potentiates capsaicin-gated currents in mouse DRG neurons (B) but has no effect in naked mole-rat neurons (C). (D and E) NGF superfusion causes sensitization of mouse DRG neurons, observed as increase in calcium influx (D), but not in naked mole-rat DRG neurons (E); high-potassium solution (40 mM KCl) was used to verify cell viability. (F) Ratios of sixth and fifth capsaicin response from (D) and (E), as labeled by arrows. Mann-Whitney *U* test was used in (B), (C), and (F) (***p* < 0.01; *****p* < 0.0001). Data are presented as mean \pm SEM.

TrkA receptor likely render the receptor hypofunctional. Thus, millions of years of evolution appear to have led to an efficient and possibly single-molecule change that disables heat hyperalgesia.

RESULTS

TRPV1 Is Not Sensitized by NGF in Naked Mole-Rat Nociceptors

We made whole-cell patch-clamp recordings from isolated mouse and naked mole-rat sensory neurons live-labeled with fluorescently tagged isolectin B4 (IB4). IB4 predominantly binds to non-peptidergic small-diameter sensory neurons in mice, while TrkA immunoreactivity is specific to peptidergic sensory neurons that do not bind IB4 (Averill et al., 1995). Immunohistochemistry confirmed that IB4-negative sensory neurons are TrkA positive in mouse and naked mole-rat, but as in the rat (Price and Flores, 2007), some naked mole-rat TrkA-positive neurons were IB4 positive (Figure 1A). We also immunostained cultured naked mole-rat sensory neurons and found that 50% (34/68 cells) were TrkA positive while 35% (24/68) were IB4 positive, and only a small proportion of TrkA-positive cells were IB4 positive (15% [5/34]). We thus focused our analysis on IB4-negative neu-

rons to increase the likelihood of recording from naked mole-rat sensory neurons that possess TrkA receptors. The ability of NGF to rapidly sensitize TRPV1 was measured by comparing capsaicin-evoked current amplitudes before and after a 5-min NGF superfusion (100 ng/mL). As in rat sensory neurons (Shu and Mendell, 1999), there was a substantial increase in the average size of the capsaicin-evoked current (>2-fold) after acute NGF treatment of IB4-negative mouse sensory neurons (Figure 1B). However, in naked mole-rat IB4-negative sensory neurons, NGF never sensitized TRPV1 currents (Figure 1C). In order to confirm these results we also performed calcium imaging on isolated mouse and naked mole-rat sensory neurons. Given that both capsaicin and calcium influx cause desensitization and tachyphylaxis of TRPV1 (Koplas et al., 1997; Lishko et al., 2007), we applied five consecutive pulses (100 nM capsaicin, 30 s pulse) in order to obtain stable calcium signals before exposing the neurons to NGF (Hanack et al., 2015), followed by the sixth capsaicin pulse (Figures 1D and 1E). In mouse sensory neurons, NGF caused robust sensitization of capsaicin responses, but no increase in calcium influx was observed in naked mole-rat sensory neurons (Figure 1F). Thus, the absence of behavioral signs of NGF-induced heat hyperalgesia in the naked mole-rat (Park et al., 2008) can be accounted for by molecular changes, intrinsic

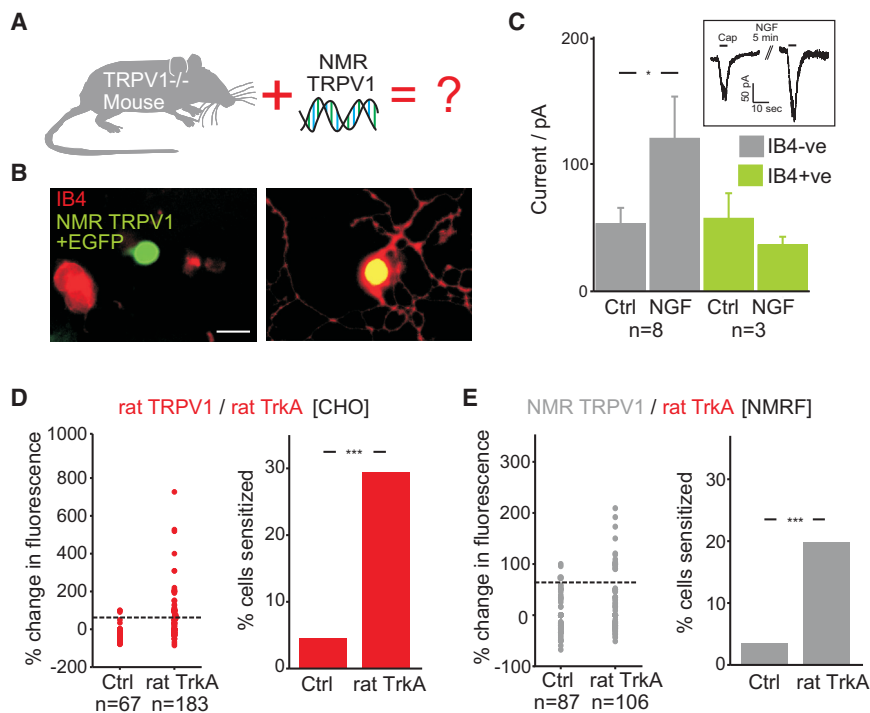


Figure 2. Naked Mole-Rat TRPV1 Currents Can Be Sensitized by NGF

(A) Naked mole-rat *Trpv1* cDNA was transfected into DRG neurons originating from *Trpv1*^{-/-} mice. (B) *Trpv1*^{-/-} DRG neurons expressing naked mole-rat TRPV1 channels were identified by co-transfection with EGFP; IB4-568 labeling allowed targeting of TrkA-positive neurons. Scale bar, 50 μ m. (C) NGF potentiates naked mole-rat TRPV1-mediated capsaicin currents in IB4-negative, but not IB4-positive, *Trpv1*^{-/-} DRG neurons. (D) In CHO cells co-expressing rat *Trpv1*/rat *TrkA*, NGF sensitized capsaicin responses, unlike in control cells. (E) Naked mole-rat fibroblast cells expressing naked mole-rat *Trpv1*/rat *TrkA* were sensitized by NGF when compared to controls. Sensitization in (D) and (E) was scored if change in [Ca²⁺]_i intensity > (mean + 2 SD) of controls (dotted lines in lefthand panels). Mann-Whitney *U* test was used in (C) and chi-square test in (D) and (E) (**p* < 0.05; ****p* < 0.001). Data in (C) are presented as mean \pm SEM.

to naked mole-rat nociceptors, that have disabled NGF sensitization of TRPV1.

The cloned naked mole-rat *TRPV1* receptor (*nmrTrpv1*) displays biophysical properties similar to its mouse counterpart with respect to proton, capsaicin, and heat gating (Smith et al., 2011). It is, however, possible that the naked mole-rat TRPV1 protein cannot be phosphorylated on critical residues that are required for full sensitization. Several conserved amino acid residues that can be phosphorylated within the TRPV1 molecule have been shown to be important for sensitization (Bhave et al., 2003; Chuang et al., 2001; Prescott and Julius, 2003; Zhang et al., 2005); however, all but one of these residues were conserved in *nmrTrpv1* (Figure S1A). Thus, Ser502 (numbering for *ratTrpv1*), a normally conserved residue involved in protein kinase C epsilon type (PKC ϵ)-mediated sensitization (Numazaki et al., 2002), was substituted by a threonine in the naked mole-rat protein. By using the phorbol-12-myristate-13-acetate ester (PMA) to activate PKC ϵ in cells transfected with *ratTrpv1*, we observed robust sensitization using calcium imaging as the readout (Figure S1B). To measure PKC ϵ sensitization of *nmrTRPV1*, we used a new naked mole-rat fibroblast cell line (Figure S1C) to enable recording of PKC ϵ -mediated sensitization of *nmrTRPV1* in its native environment, which was robustly observed (Figure S1D). We also generated a naked mole-rat *TRPV1*^{T502S} mutant that was also sensitized by PMA in naked mole-rat fibroblast cell lines (Figure S1E) and conclude that in terms of TRPV1 sensitization, threonine is functionally equivalent to serine at position 502.

To demonstrate more directly that naked mole-rat TRPV1 is fully capable of being sensitized, we expressed it in mouse sensory neurons from *Trpv1*^{-/-} mice. Using an Alexa-Fluor-568-

conjugated IB4, mouse *Trpv1*^{-/-} IB4-negative sensory neurons were selected by their green fluorescence after transfection with plasmids encoding EGFP and the naked mole-rat *Trpv1* cDNA (Figures 2A and 2B). We used whole-cell patch-clamp electrophysiology to demonstrate that capsaicin-evoked currents are present in transfected *Trpv1*^{-/-} sensory neurons and that these currents could be sensitized by NGF (Figure 2C). In contrast, no sensitization of the capsaicin current in mouse IB4-positive sensory neurons was observed presumably because of the absence of TrkA in these cells (Figures 2C and 1A). Transfected *Trpv1*^{-/-} sensory neurons had heat-gated currents with an activation threshold of 44.4°C \pm 0.7°C (*n* = 5) and pH-gated currents sensitive to ruthenium red (Figure S1F). Heat-activated currents are reported to be otherwise rare in *Trpv1*^{-/-} sensory neurons (Caterina et al., 2000). Thus, the naked mole-rat TRPV1 protein can rescue capsaicin and heat sensitivity in *Trpv1*^{-/-} sensory neurons with a heat-activation threshold concomitant with the heat-activation threshold of *nmrTRPV1* (Smith et al., 2011) and is fully capable of NGF initiated sensitization in the mouse cellular context.

Rapid TRPV1 sensitization via NGF activation of TrkA receptors can be reconstituted in human and animal-derived cell lines as well as *Xenopus laevis* oocytes (Bonnington and McNaughton, 2003; Prescott and Julius, 2003; Zhang et al., 2005). It is possible that TRPV1 sensitization by NGF cannot take place in the naked mole-rat cellular context. We used calcium imaging to measure TRPV1 sensitization after Chinese hamster ovary (CHO) cells were transfected with *ratTrpv1*/*ratTrkA*. In control experiments, *ratTrkA* was either not transfected or buffer instead of NGF was superfused between the capsaicin pulses. NGF produced a robust sensitization with 29.5% of capsaicin-responsive CHO cells showing increased calcium signals post-NGF compared to just 4.5% in controls (Figure 2D). Similarly, using naked mole-rat fibroblast cells

co-transfected with *nmrTrpv1/ratTrkA*, 19.8% of capsaicin-responsive naked mole-rat fibroblast cells showed increased calcium signals post-NGF compared to just 3.5% in controls (Figure 2E). These data indicate that naked mole-rat cells possess the necessary signaling components for TRPV1 sensitization.

Naked Mole-Rat TrkA Is Hypofunctional

We cloned the naked mole-rat *TrkA* cDNA from mRNA isolated from sensory neurons (*nmrTrkA*). The *nmrTrkA* sequence was identical to that predicted from the naked mole-rat genome assembly (Keane et al., 2014; Kim et al., 2011). The predicted naked mole-rat *TrkA* peptide sequence was aligned with orthologous sequences from 26 other mammalian species (Figure S2). There was significant sequence divergence in the extracellular *TrkA* domains, including the juxtamembrane NGF-binding domain; however, the intracellular sequences within the kinase domain were highly conserved (Figure S2B). All tyrosine residues important for receptor activation were conserved in all the species, including the naked mole-rat. We reasoned that at least some of the amino acid variants in the kinase domain of *nmrTrkA* may be common variants found in African mole-rats (family Bathyergidae). In order to screen for such variants, we obtained *TrkA* sequences from five further African mole-rat species: the Damaraland mole-rat (*Fukomys damarensis*), the Mashona mole-rat (*Fukomys darlingi*), the giant mole-rat (*Fukomys mechowii*), the Natal mole-rat (*Cryptomys hottentotus natalensis*), and Emin's mole-rat (*Heliphobius emini*) (Figure S3A). We used genomic DNA from these species to PCR amplify the exonic regions of the *TrkA* gene, guided by variants found in *nmrTrkA*. However, we also assembled *TrkA* transcripts from published RNA sequencing (RNA-seq) data from African mole-rat species (Davies et al., 2015). In addition, we obtained RNA from the brains of three Mashona mole-rats and performed RNA-seq followed by de novo transcriptome assembly (Table S1). An African mole-rat phylogeny was constructed including the new transcriptome data from the Mashona mole-rat (Figure S3C), and this was in close agreement with previous analyses that had not included this species (Davies et al., 2015). Alignment of the available predicted *TrkA* amino acid sequences from African mole-rats revealed that the *nmrTrkA* kinase domain has accumulated at least three amino acid variants that are either absent or rare in the animal kingdom, including African mole-rats (Figure S3B). There was just one amino acid change that appeared to be unique to naked mole-rat, which was a leucine (rat) to cysteine substitution at position 774 (Figure S2B). The accumulation of amino acid variants in the *nmrTrkA* kinase domain encouraged us to carry out a functional analysis of the ability of this receptor to participate in nociceptor sensitization. To do this, we tested the ability of the naked mole-rat *TrkA* receptor to sensitize TRPV1 using electrophysiology with *X. laevis* oocytes as the heterologous expression system. Oocytes were injected with a *ratTrpv1* cRNA and cRNAs coding for either *ratTrkA* or *nmrTrkA*. We observed that 1 μ M capsaicin causes substantial and long-lasting desensitization of TRPV1 currents in oocytes and thus decided to record proton-gated TRPV1 currents to quantify NGF sensitization, as others have done (Zhang et al., 2005). Using a two-electrode voltage clamp, we showed that

an acidic stimulus (pH 5.8) produced robust inward currents in TRPV1-expressing oocytes that were absent in non-injected oocytes (data not shown). In oocytes injected with *ratTrkA* and *ratTrpv1* cRNA, superfusion of NGF (100 ng/mL, 5 min) caused a robust sensitization of acid-gated currents (Figure 3B). However, the same NGF concentration produced a significantly smaller sensitization of TRPV1 currents in oocytes injected with *nmrTrkA* and *ratTrpv1* cRNA (Figures 3B and 3E). Comparable amounts of rat and naked mole-rat *TrkA* protein were present in membranes isolated from *X. laevis* oocytes (Figure 3C), indicating that differences in *TrkA* protein levels was unlikely to account for the reduced TRPV1 sensitization. We next varied NGF concentration (1–1,000 ng/mL) but kept the superfusion time constant (5 min). *TrkA* is a high-affinity NGF receptor with a dissociation constant K_d of less than 10^{-9} M (Kaplan et al., 1991; Klein et al., 1991). When oocytes were stimulated with 1,000 ng/mL NGF, activation of the naked mole-rat *TrkA* receptor produced a degree of sensitization similar to that observed with rat *TrkA* (Figure 3C). These results strongly suggest that the naked mole-rat *TrkA* molecule is less efficient at initiating sensitization with NGF concentrations of \sim 100 ng/mL, which was shown to be saturating in adult rat sensory neurons (Shu and Mendell, 1999). It is conceivable that recombinant human NGF used in this study (rhNGF) displays stronger binding affinity to rat *TrkA* than to the naked-mole-rat *TrkA*. To test this idea, we cloned chimeric *TrkA* receptors containing the N-terminal, extracellular part of the receptor from rat *TrkA* together with the transmembrane domain and entire intracellular kinase domain from the naked mole-rat molecule (Figures 3D and 3E). HEK293 cells were transiently transfected with either rat or chimeric *TrkA* construct to assess NGF-stimulated *TrkA* activation (Figure S4). An antibody raised against extracellular rat *TrkA* domain was used to measure the total level of *TrkA* protein in cell lysates (total *TrkA*), and two antibodies that recognize phosphorylated tyrosine residues in the *TrkA* kinase domain were employed to study receptor activation. Anti-phospho-*TrkA* (Tyr674/675; numbering for human *TrkA*) was used to measure the phosphorylation levels of the activation loop tyrosines (Segal and Greenberg, 1996; Segal et al., 1996), and an anti-phospho-*TrkA* (Tyr490) was used that recognizes the activated putative Shc binding site (Obermeier et al., 1993a). NGF stimulation triggered rapid phosphorylation of Tyr674/675 in rat *TrkA*, but not in chimeric *TrkA* (Figures S4A and S4B). In contrast to rat *TrkA*, NGF treatment did not have any effect on activation of Tyr674/675 in the chimeric *TrkA* receptor. However, the Tyr674/675 residues in both chimeric *TrkA* and rat *TrkA* displayed strong basal receptor phosphorylation in the absence of NGF, probably triggered by receptor dimerization events due to overexpression. This observation is in agreement with previous findings that an antibody against the *TrkA* extracellular domain can itself crosslink two receptors, causing their activation in PC12 cells (Clary et al., 1994; Hempstead et al., 1992). NGF triggered increased phosphorylation of the Tyr490 residue in the rat *TrkA* molecule after 1 min but did not have any apparent effect on the phosphorylation level of the chimeric *TrkA* Tyr490 residue (Figures S4C and S4D). Next, we tested chimeric *TrkA* in the context of NGF-mediated TRPV1 sensitization. Proton acid-gated TRPV1 currents in *X. laevis* oocytes co-expressing chimeric *TrkA* could only be

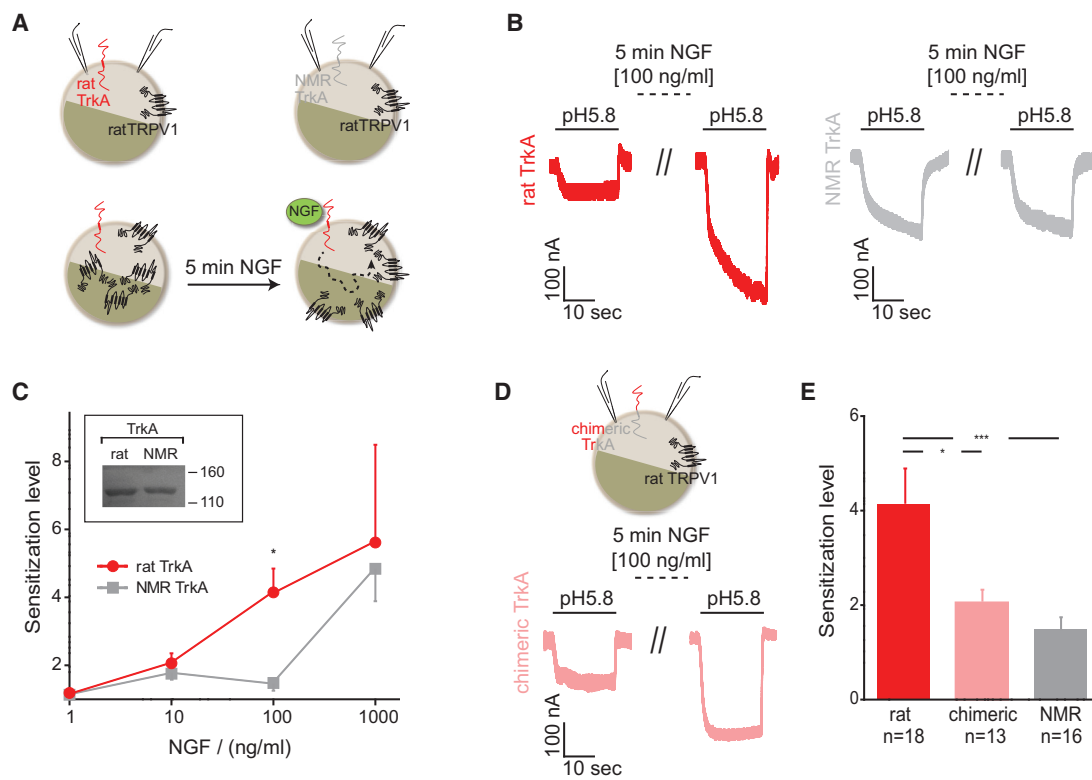


Figure 3. Naked Mole-Rat TrkA Is Impaired in TRPV1 Current Potentiation

(A) Schematic representation of the transfection and recording conditions used.

(B) NGF causes substantial sensitization of proton-gated rat TRPV1 currents when signaling through rat TrkA, but this effect is reduced in the oocytes co-expressing the naked mole-rat TrkA receptor.

(C) Sensitization levels across NGF concentrations were calculated as the ratio of a current immediately after and before NGF superfusion. High NGF concentration rescues the sensitization through NGF TrkA. For all the measurements, at least three pH stimuli were applied before the NGF superfusion in order to obtain the stable current responses, while at least two acid-gated currents were recorded post-NGF application. Between 2 and 18 oocytes were recorded for every NGF concentration. Inset: ten oocytes, injected with equal concentration of rat or naked mole-rat *TrkA* cRNA, were lysed, and pelleted membranes were subjected to western blotting. Prior to blotting, the protein concentration was measured to ensure equal sample loading. TrkA is expressed as a 140-kDa protein. (D and E) 100 ng/mL NGF potentiated acid-gated TRPV1 currents recorded in *X. laevis* oocytes via chimeric TrkA (D), but the sensitization level was significantly smaller than for rat TrkA and not different from the NMR TrkA receptor quantified in (E).

Two-way ANOVA with Sidak's multiple comparison was used in (C), and one-way ANOVA with Bonferroni's multiple comparison test was used in (E) ($p < 0.05$; $***p < 0.001$). Data are presented as mean \pm SEM, except in (C) for NMR TrkA at 1 ng/mL NGF (only mean current plotted), where two oocytes were recorded.

moderately sensitized with 100 ng/mL NGF; indeed, the mean level of sensitization observed was not significantly larger than that found with the full-length *nmrTrkA* (Figure 3E). In contrast, sensitization of TRPV1 proton currents by NGF-stimulated oocytes co-expressing *ratTrkA* was at least twice as large as with full-length *nmrTrkA* or chimeric receptors. These results strongly suggest that a hypo-functional naked mole-rat TrkA kinase domain underlies the lack of TRPV1 sensitization in this species.

Quantitative Proteomics Reveals Hypofunctional Downstream Signaling of the *nmrTrkA* Intracellular Domain

A quantitative proteomics approach was used that combined HEK293 cell stable isotope labeling by amino acids in cell culture (SILAC) (Ong et al., 2002) with high-resolution liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Heavy-stable isotope (Lys-8 and Arg-10)-labeled HEK cells tran-

siently expressing rat or chimeric TrkA were stimulated with murine NGF (100 ng/mL) for 10 min and light-stable isotope (Lys-0 and Arg-0) cells were left untreated (Figure 4A) (Olsen et al., 2006). Following stimulation, cells were lysed, and equal amounts of protein were used for each SILAC pair (Figure 4A). Titanium dioxide (TiO₂) chromatography was used for phosphopeptide enrichment, and peptides were analyzed with LC-MS/MS. Typically, phosphopeptides were sequenced several times in different forms (such as oxidized methionine or missed tryptic cleavage), and overall, a similar number of phosphopeptide sites were quantified and identified in cells transfected with rat TrkA or chimeric TrkA (Figure 4B), indicating a similar overall number of phosphorylated proteins in each condition. Class I phosphopeptide sites comprise those residues with the highest localization probability for the phospho-group (>0.75); that is, the sum of probabilities of other potential sites is less than 0.25. From more than 2,000 identified and quantified class I phospho-sites,

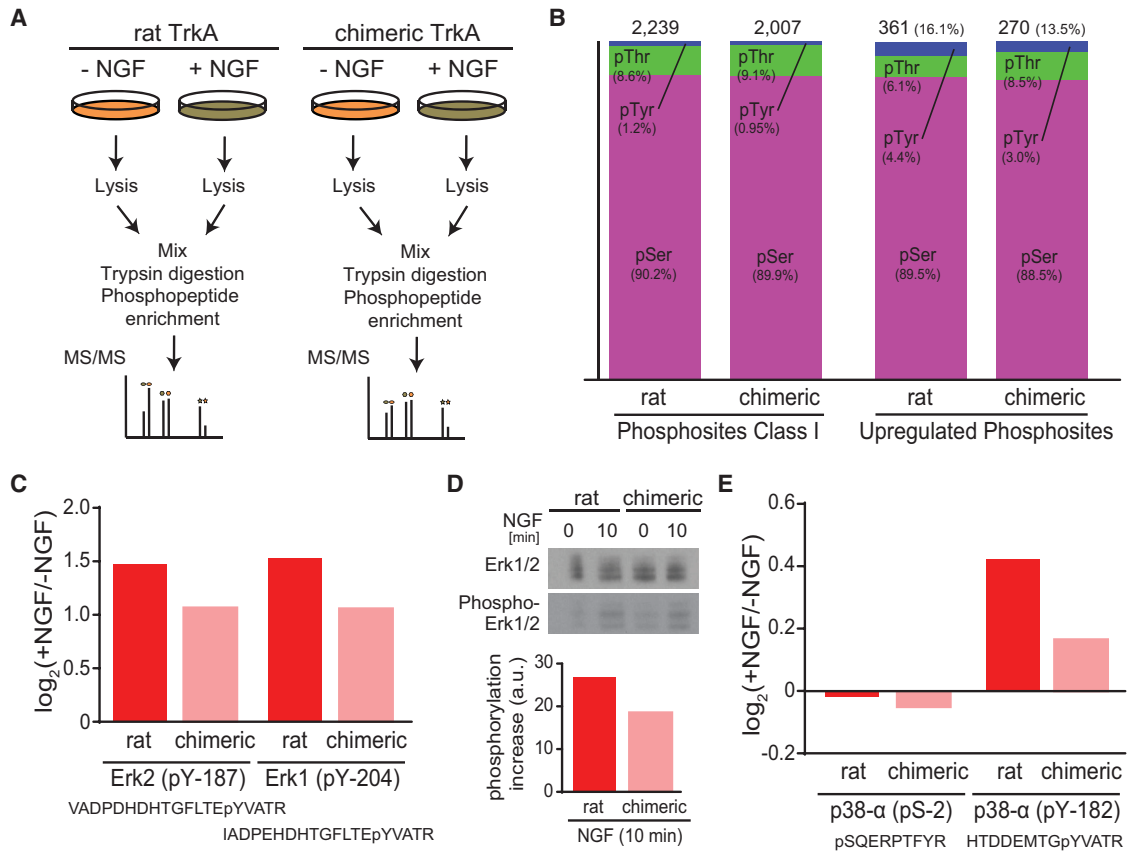


Figure 4. Quantitative Proteomics Reveals Hypofunctional Signaling of the nmrTrkA Intracellular Domain

(A) Overview of the SILAC experiment, performed as a biological duplicate for both receptors. Following the trypsin digestion, samples were enriched for phosphopeptides on TiO_2 columns.

(B) Similar number of phosphopeptides were identified and quantified in both rat and chimeric TrkA samples, but significantly more peptides were upregulated when signaling through rat TrkA than through chimeric TrkA (Fisher's exact test, two-tailed $p = 0.0155$).

(C and D) Activation of rat TrkA triggers stronger phosphorylation of Erk1/2 key regulatory residues than activation of chimeric TrkA.

(E) Regulatory tyrosine-182 residue of p38- α kinase shows stronger phosphorylation when signaling through rat TrkA, but NGF activation does not change phosphorylation levels of the serine-2 residue.

Data in (C)–(E) are presented as mean values from two experiments.

the distribution of phosphoserine (pSer), phosphothreonine (pThr), and phosphotyrosine (pTyr) sites observed in cells transfected with rat or chimeric TrkA was similar to distributions reported previously with cells stimulated with NGF or epidermal growth factor (EGF) (Emdal et al., 2015; Olsen et al., 2006). We next examined the NGF-upregulated phospho-sites and found that significantly more phosphopeptides were upregulated in NGF-treated cells with rat TrkA (361/2,239 [16.8%]) compared to cells with chimeric TrkA (270/2,007 [13.5%]; Figure 4B).

Analysis of upregulated phospho-site sequence motifs was used to extract over-represented and enriched sequence patterns (Chou and Schwartz, 2011; Schwartz and Gygi, 2005). NGF stimulation was associated with the upregulation of a similar pattern of sequence motifs surrounding pSer residues in cells with rat or chimeric TrkA (Figure S5). However, stimulation of the rat TrkA receptor was associated with a more substantial enrichment of proline-containing motifs compared to chimeric TrkA, which is an indicator of stronger

activation of MAPK/CDK protein families (Amanchy et al., 2007).

An additional quantitative proteomics experiment revealed that there was a stronger activation of specific phosphopeptides from Erk2 (MAPK1, pTyr-187) and Erk1 (MAPK3, pTyr-204) (Critton et al., 2008; Sacco et al., 2009) after stimulation of the cells expressing rat TrkA compared to chimeric TrkA (Figure 4C). In addition, western blotting for phosphorylated Erk in HEK293 cells transfected with rat or chimeric TrkA after NGF stimulation revealed reduced levels of phospho-Erk protein after stimulation of the chimeric receptor (Figure 4D). We could also identify and quantify the changes in phosphopeptides from p38- α (MAPK14), a kinase involved in TRPV1 regulation in sensory neurons (Ji et al., 2002; Raingeaud et al., 1995). We observed a stronger increase in the abundance of p38- α derived-phosphopeptides containing the pTyr182 residue after stimulation of rat TrkA compared to chimeric TrkA (Figure 4E). A phosphoserine residue on the same protein (pSer2) (Olsen et al., 2010) did not

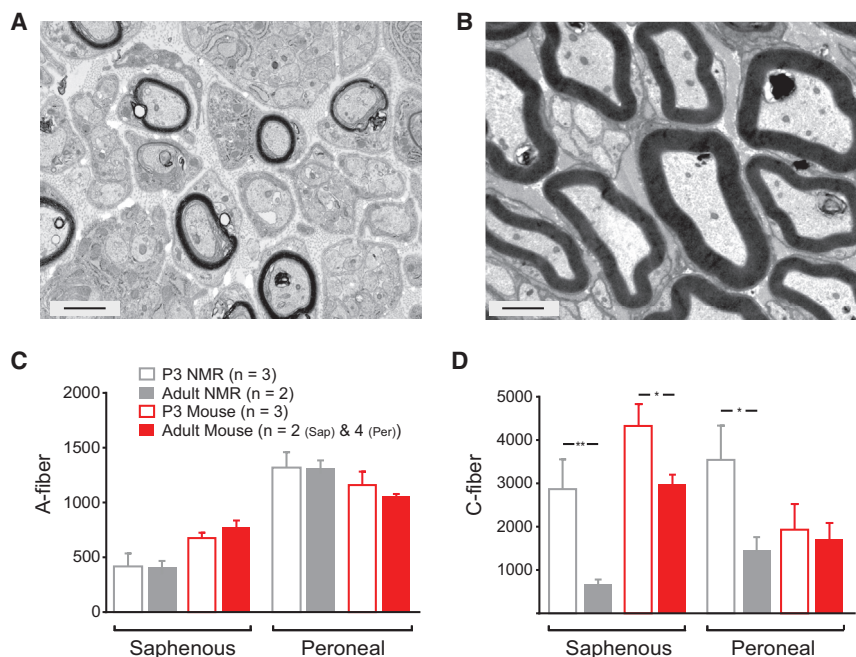


Figure 5. Naked Mole-Rat Pups Have More C-Fibers in Peripheral Nerves than Adults

(A and B) Example electron micrograph of the saphenous nerve of an NMR P3 pup (A) and an adult animal (B). Different myelination stages of single A-fibers and C-fibers within Remak bundles are visible; scale bar, 1 μ m.

(C) Numbers of fibers with detectable myelination were comparable for neonatal and adult nerves in both naked mole-rat and mouse.

(D) Quantification of C-fiber number for the saphenous and peroneal nerve in the pup compared to the adult nerve from naked mole-rat and mouse. For comparison, naked mole-rat adult data were taken from [St. John Smith et al. \(2012\)](#) and mouse adult data were taken from [Moshourab et al. \(2013\)](#) and [Robertson and Sima \(1980\)](#) for mouse saphenous and common peroneal nerve, respectively.

Numbers in (C) and (D) indicate the number of animals used for quantification (two nerves per animal). Mann-Whitney *U* test was used (**p* < 0.05; ***p* < 0.01). Data are presented as mean \pm SEM.

show any significant change in either condition after NGF stimulation indicating specificity of NGF-mediated activation (Figure 4E).

Developmental Consequences of Hypofunctional TrkA in the Naked Mole-Rat

NGF-TrkA signaling is essential for the survival of embryonic sensory neurons ([Lallemend and Erfors, 2012](#); [Lewin and Barde, 1996](#)). Adult naked mole-rats have a striking paucity of C-fibers in cutaneous nerves ([St John Smith et al., 2012](#)), a feature that is reminiscent of NGF/TrkA loss of function in humans and mice ([Crowley et al., 1994](#); [Indo et al., 1996](#)). We thus used transmission electron microscopy to quantify the numbers of myelinated and unmyelinated fibers in peripheral nerves of postnatal day 3 (P3) naked mole-rats and mice (Figures 5A and 5B). We compared the numbers of myelinated (or myelinating) axons in the saphenous and common peroneal nerves in neonates with the published values for adult mice and naked mole-rats using identical methods. We found that the number of unmyelinated C-fibers counted in cross-sections from the purely cutaneous saphenous nerve and the mixed common peroneal nerve from naked mole-rats was between 2- and 3.5-fold higher than the number observed in adult nerves (Figure 5D). However, the number of unmyelinated fibers found in the mouse common peroneal nerve did not change between P3 and adult mole-rats, although there was a small attrition of C-fibers from the saphenous nerve (Figure 5D). In contrast, although the peripheral nerves of P3 naked mole-rats and mice are still undergoing myelination (Figures 5A and 5B), the number of fibers with a myelin sheath (A-fibers) was not different between nerves from the neonate and adult (Figure 5C). These data suggested that there is substantial loss of unmyelinated axons from cutaneous and mixed peripheral nerves of naked mole-rats between P3 and adulthood.

DISCUSSION

We dissected the molecular mechanism that underlies the absence of thermal hyperalgesia in the African naked mole-rat (*H. glaber*) ([Park et al., 2008](#)). NGF is central player in the generation of thermal hyperalgesia and acts via its receptor TrkA to initiate hyperalgesia in a TRPV1-dependent manner ([Bonnington and McNaughton, 2003](#); [Chuang et al., 2001](#); [Lewin et al., 2014](#)). We have shown that lack of heat hyperalgesia in the naked mole-rat is associated with absence of NGF-induced TRPV1 sensitization in sensory neurons. Our data indicate that the key molecular change in the signal transduction pathway from NGF to hyperalgesia is a unique but minimal sequence change in the naked mole-rat TrkA molecule. We provide evidence that between one and three unique amino acid substitutions within the kinase domain make the naked mole-rat TrkA receptor less efficient at engaging downstream signal transduction, including members of the MAPK family of effectors. Efficient NGF signaling is also a prerequisite for the survival and terminal branching of embryonic sensory neurons in the mouse ([Crowley et al., 1994](#); [Patel et al., 2000](#)). Interestingly, a hypofunctional TrkA receptor in the naked mole-rat is associated with a striking paucity of unmyelinated C-fibers in adult peripheral nerves ([St John Smith et al., 2012](#)). A comparative anatomical study of six other African mole-rat species (for which TrkA sequences were obtained here) indicated that the C-fiber deficit appears to be unique to naked mole-rats ([St John Smith et al., 2012](#)). Even though the Mashona mole-rat (*F. darlingi*) shares two out of the three unique amino acid variants found in TrkA kinase domain of the naked mole-rat receptor (Figure S3B), it does not lack C-fibers ([St John Smith et al., 2012](#)). We thus postulate that hypofunctional TrkA signaling in vivo may lead to a loss of C-fibers in naked mole-rats. However, newborn naked mole-rats were found to have

many more C-fibers in peripheral nerves than adults. This finding suggests that C-fibers in the naked mole-rat are lost between P3 and adulthood, perhaps as a consequence of hypofunctional TrkA signaling.

Among vertebrate receptors, the TrkA receptor displays the strictest conservation in the intracellular kinase domain (Figure S2). Using chimeric TrkA receptors (rat extracellular/naked mole-rat intracellular), we could show directly that the reduced ability of the naked mole-rat TrkA receptor to sensitize TRPV1 currents is likely localized to the kinase domain (Figure 3). Indeed, biochemical experiments demonstrated a striking reduction in signaling capacity in terms of ligand-dependent tyrosine phosphorylation (Figure S4); however, all the important tyrosine residues in the kinase domain are conserved in the naked mole-rat TrkA molecule (Figure S2). We speculate that insertion of a cysteine for a leucine at position 774 in the naked mole-rat TrkA receptor may alter the efficiency of phosphorylation or recognition of the flanking tyrosine's Tyr751 and Tyr785. Tyrosine 751 has been implicated in binding of the p85 subunit of phosphoinositide 3-kinase (Obermeier et al., 1993b), and Tyr785 serves as a major and selective interaction site for phosphoinositide phospholipase C- γ (Obermeier et al., 1993a). It is of course also possible that accumulated effects of the other variants that are not specific to naked mole-rat TrkA (Figure S3B) contribute to the reduction in receptor signaling we have observed.

Our data strongly suggest that molecular changes in naked mole-rat TrkA molecule alter signal transduction efficiency. Ligand concentration of 100 ng/mL produced almost maximal sensitization of TRPV1 in our oocyte expression system, a similar dose dependence to that found for capsaicin current sensitization in rat sensory neurons (Shu and Mendell, 1999). In contrast, NGF stimulation of chimeric TrkA receptor produced little sensitization of TRPV1 currents at 100 ng/mL but normal sensitization at 1,000 ng/mL (Figure 3C). The maintained efficacy of the naked mole-rat TrkA receptor at very high NGF concentrations is consistent with our previous observation that NGF (500 ng/mL) promotes neurite outgrowth of both mouse and naked mole-rat sensory neurons in culture (Park et al., 2008). However, it is well known that orders-of-magnitude lower concentrations of NGF (<1 ng/mL) are capable of promoting maximal neuronal survival or neurite outgrowth in developing neurons (Davies et al., 1993; Vaillant et al., 2002; Ye et al., 2003). Using a high-resolution quantitative proteomics approach, we found that 10 min after NGF stimulation with 100 ng/mL, there were subtle but significant differences in upregulated phosphopeptides between rat TrkA and a chimeric TrkA containing the naked mole-rat intracellular domain. We obtained evidence of reduced pTyr on peptides belonging to MAPK proteins, including p38 α , which has been directly implicated in the sensitization of TRPV1 (Ji et al., 2002).

Surprisingly, naked mole-rat pups do not show the deficit in C-fibers that we had observed in adult animals (Figure 5). It thus appears that the signaling capacity of the naked mole-rat TrkA is sufficient to support the survival and functional development of sensory neurons during embryonic development (Crowley et al., 1994; Lechner et al., 2009). NGF is functionally important for the maintenance of mature sensory neurons (Lewin

et al., 2014), but rodents exposed to NGF-function blocking antibodies exhibit death of sympathetic neurons, but probably not sensory neurons (Gorin and Johnson, 1980; Lewin et al., 1992; Ruberti et al., 2000). The concentrations of NGF that robustly sensitize TRPV1 in adult neurons are clearly much higher than those needed to support embryonic survival (see above). It is thus conceivable that the molecular changes in the naked mole-rat TrkA receptor that we describe are more relevant to physiological processes that follow strong receptor stimulation. In this context it is interesting to note that NGF signaling in adult naked mole-rat is still capable of producing mechanical hyperalgesia, a process that does not involve TRPV1 (Lewin et al., 2014). It is possible that hypofunctional TrkA signaling leads to the loss of sensory neurons in naked mole-rats after birth. Naked mole-rats have an extraordinarily long gestation period of ~70 days and can live for up to 32 years (Jarvis, 1991; Sanchez et al., 2015). It is thus feasible that developmental events that occur just after birth, like the loss of TrkA expression in approximately half of the nociceptors (Bennett et al., 1998; Molliver et al., 1997), occur over a more protracted period in the naked mole-rat. In this context, it is important to note that some nociceptors in rats and mice are still dependent on NGF for survival for a few days after birth (Crowley et al., 1994; Lewin et al., 1992). We find that the paucity of C-fibers in cutaneous nerves is correlated with molecular changes in the TrkA receptor associated with reduced signaling (Figure S4). However, it is still possible that effects of other as-yet-unknown gene variants in the naked mole-rat potentiate the effects of the TrkA variants to promote postnatal nociceptor loss.

In summary, we provide evidence that evolution has selected for a single-molecule change in the naked mole-rat NGF receptor TrkA that is sufficient to abolish heat hyperalgesia in this species. Mutations in the *trkA* gene are highly detrimental in humans, but here we show that evolution has selected for sequence change(s) in the naked mole-rat gene that are not only functionally powerful but also compatible with species survival and continued fitness. We speculate that heat hyperalgesia is not an essential phenotypic attribute for the naked mole-rat that is adapted to a subterranean habitat in equatorial East Africa, where temperatures have remained constant for millions of years. Other African mole-rat species have apparently not dispensed with efficient TrkA signaling, and we speculate that one reason for this is that the naked mole-rat is probably the most energetically challenged species in this family (Bennett and Faulkes, 2000). Thus, naked mole-rats can make do with a stripped-down nociceptive system, equipped with fewer C-fibers, that requires less energy but is sufficient for acute nociception and mechanical hyperalgesia following injury (Park et al., 2008). Our study illustrates how evolution can select for mechanistically novel single-molecule changes that exert dramatic phenotypic effects but are compatible with the maintenance of species fitness.

EXPERIMENTAL PROCEDURES

DRG Neuron and Cell Culture

Animal housing, care, and protocols for euthanasia were approved by German federal authorities (State of Berlin). Dorsal root ganglia (DRG) neurons were

prepared from both naked mole-rat and mouse as described previously (Park et al., 2008) and plated onto glass coverslips plated with poly-L-lysine (PLL; 200 mg/mL) and laminin (20 μ g/mL). CHO and naked mole-rat fibroblast cells were cultured in F12-Ham medium (Life Technologies) and incubated at 37°C in 5% CO₂. For electrophysiology experiments, cells were plated onto PLL-coated plastic dishes and the following day transfected with Lipofectamine (Invitrogen).

Electrophysiology

Recordings from DRG neurons took place after a 10- to 20-min incubation with either IB4-Alexa 488 or IB4-Alexa Fluor 568 (Invitrogen). Whole-cell recordings were made using pipettes (3–6 M Ω resistance) pulled with a Flaming-Brown puller (Sutter Instruments). Extracellular solution contained 140 mM NaCl, 1 mM MgCl₂, 2 mM CaCl₂, 4 mM KCl, 4 mM glucose, and 10 mM HEPES (pH 7.4) with NaOH. Electrodes were filled with 110 mM KCl, 10 mM NaCl, 1 mM MgCl₂, 1 mM EGTA, and 10 mM HEPES (pH 7.3). Solutions were applied and heated using a gravity-driven multi-barrel perfusion system (WAS-02) (Dittert et al., 2006).

X. laevis defolliculated oocytes (stage V or VI) were purchased from EcoCyte Bioscience. Each oocyte was injected in Barth solution using the Nanoject II Auto-Nanoliter Injector (Drummond) with 32.2 nL cRNA mix. Two-electrode voltage-clamp recordings were performed at room temperature 3–5 days after injection using a GeneClamp500B Amplifier, Digidata 1322A, and pClamp 8.0 Software (Axon Instruments). Additional details are available in [Supplemental Experimental Procedures](#).

Molecular Biology and RNA Sequencing

Cloning of naked mole-rat Trpv1 was described before (Smith et al., 2011). In order to clone naked mole-rat TrkA, total RNA was isolated from DRGs with TRIzol (Life Technologies) and dissolved in 30 μ L RNase-free water. 1–3 μ g total RNA and oligo(dT) and random hexamers (BioTeZ) were used for cDNA synthesis using SuperScript III Reverse Transcriptase (Life Technologies).

To sequence the coding DNA sequence (CDS) for the TrkA intracellular kinase domain of other African mole-rat species, primers specific for NMR TrkA were used to amplify exons 12–17 from species' genomic DNA. Five mole-rats representative of the Bathyergidae family were used: Giant (*F. mechowii*), Damaraland (*Fukomys damarensis*), Mashona (*Fukomys darlingi*), Natal (*Cryptomys hottentotus natalensis*), and Emin's (*Heliophobius emini*) mole-rats. RNA-seq data for *F. darlingi* were generated from three brain samples using paired-end, strand-specific (dUTP) libraries that were sequenced on an Illumina HiSeq2000 platform. The accession number for the annotated transcriptome and the sequencing reads from the Mashona mole-rat reported in this paper is NCBI: PRJNA303968. Additional details are available in [Supplemental Experimental Procedures](#).

Immunocytochemistry and Calcium Imaging

Standard immunohistochemistry and immunocytochemistry protocols on NMR and mouse DRGs were used using an anti-TrkA antibody (kind gift from L.F. Reichardt, UCSF) and IB4-488. Immunofluorescent images were examined with a Leica DM 5000B microscope and MetaVue software (Visitron).

Calcium imaging was conducted as described previously (Milenkovic et al., 2007). Standard Fura-2 ratiometric calcium imaging was conducted to measure responses to capsaicin in CHO and naked mole-rat fibroblast cells transfected with rat TRPV1 and NMR TRPV1, respectively, with or without rTrkA. An inverted microscope (Zeiss Observer A1) equipped with the MetaFluor photonics imaging system, including Polychromator V or DG4 (Sutter Instruments), and a CoolSNAP ES camera (Visitron) was used for cell imaging. Additional details are available in [Supplemental Experimental Procedures](#).

MS-Based Protein Quantification Using SILAC

SILAC-labeled HEK293 cells were transfected with TrkA constructs (rat or chimeric) and pEGFP plasmid (5:1) with polyethylenimine. 24 hr after transfection, cells were serum starved and stimulated for 10 min with 100 ng/mL NGF (murine 2.5S, Promega) or left untreated. Equal amounts of protein from each SILAC pair were mixed together. Protein mixtures were reduced with DTT, al-

kylated with iodoacetamide, pre-digested with Lysyl endopeptidase (LysC, Wako), and subjected to trypsin digestion overnight. Peptides were purified from stop-and-go extraction (STAGE) tips. Phosphopeptide enrichment was performed on 0.5 mg TiO₂ beads. Phosphopeptides were separated on a monolithic column (100 μ m inner diameter \times 2,000 mm, MonoCap C18 High Resolution 2000 [GL Sciences]; kindly provided by Dr. Yasushi Ishihama [Kyoto University]). The Q Exactive instrument (Thermo Fisher Scientific) was operated in the data-dependent mode, and MaxQuant software was used to identify and quantify proteins. MS/MS spectra were searched using the Andromeda search engine. Additional details are available in [Supplemental Experimental Procedures](#).

ACCESSION NUMBERS

The accession number for the annotated transcriptome and the sequencing reads from the Mashona mole-rat reported in this paper is NCBI: PRJNA303968.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2016.09.035>.

AUTHOR CONTRIBUTIONS

D.O., E.S.J.S., M.M., and G.R.L. designed experiments; D.O., E.S.J.S., and M.M. performed cloning, electrophysiology, and imaging experiments. J.H. and D.O. performed biochemical studies. D.O. and M.S. designed, analyzed, and performed quantitative proteomic studies of TrkA signaling. O.E. and J.R. isolated mRNA and performed transcriptome assemblies and bioinformatics analysis. D.O., J.R., N.B., and G.R.L. collected Mashona mole-rat samples. C.G.F. donated DNA samples from African mole-rats for TrkA sequence analysis. D.O., E.S.J.S., and G.R.L. wrote the manuscript with input from all authors.

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