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TNF-α/TNFR1 Signaling Is Required for the Development and Function of Primary Nociceptors

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SUMMARY

Primary nociceptors relay painful touch information from the periphery to the spinal cord. Although it is established that signals generated by receptor tyrosine kinases TrkA and Ret coordinate the development of distinct nociceptive circuits, mechanisms modulating TrkA or Ret pathways in developing nociceptors are unknown. We have identified tumor necrosis factor (TNF) receptor 1 (TNFR1) as a critical modifier of TrkA and Ret signaling in peptidergic and nonpeptidergic nociceptors. Specifically, TrkA+ peptidergic nociceptors require TNF-α-TNFR1 forward signaling to suppress nerve growth factor (NGF)-mediated neurite growth, survival, excitability, and differentiation. Conversely, TNFR1-TNF-α reverse signaling augments the neurite growth and excitability of Ret+ nonpeptidergic nociceptors. The developmental and functional nociceptive defects associated with loss of TNFR1 signaling manifest behaviorally as lower pain thresholds caused by increased sensitivity to NGF. Thus, TNFR1 exerts a dual role in nociceptor information processing by suppressing TrkA and enhancing Ret signaling in peptidergic and nonpeptidergic nociceptors, respectively.

INTRODUCTION

The ability to discriminate between innocuous and painful touch is critical for survival. Nociceptors are polymodal sensory neurons that relay information about noxious tactile cues from peripheral targets to the spinal cord (Julius and Basbaum, 2001; Craig, 2003; Basbaum et al., 2009). Importantly, nociceptors are defined by their high excitability thresholds for thermal, mechanical, or chemical stimuli, meaning that pain is perceived only when these stimuli are potent enough to trigger action po-

tentials (Woolf and Salter, 2000; Hunt and Mantyh, 2001). Critical properties of nociceptors that govern the perceived intensity of a painful stimulus include density of peripheral target innervation, proper targeting of central projections, and high excitability thresholds (Pezet and McMahon, 2006). It follows that if any of these components were enhanced or attenuated then the perception of pain would be altered.

Target-derived nerve growth factor (NGF) initiates signaling pathways critical for tuning nociceptor sensitivity through the regulation of survival, neurite growth, and excitability (Crowley et al., 1994; Patel et al., 2000; Chuang et al., 2001). Peripheral targets such as the skin release NGF during development, around the time that newly born nociceptors begin to innervate peripheral targets (Marmigère and Ernfors, 2007; Lallemend and Ernfors, 2012). Secreted NGF binds to a receptor tyrosine kinase (RTK), TrkA, on developing nociceptors to transduce intracellular signals that are required for survival and target innervation (Crowley et al., 1994; Patel et al., 2000). Deletion of genes encoding NGF or TrkA causes the death of all nociceptors during development and, as a result, pain insensitivity (Verhoeven et al., 2006; Indo et al., 1996). Conversely, hyperactivation of NGF-TrkA signaling skews pain thresholds toward hypersensitivity, causing hyperalgesia or allodynia (Lewin et al., 1993; Woolf and Salter, 2000; Costigan et al., 2009; Mantyh et al., 2011). Thus, NGF signaling must be tightly controlled during development such that painful stimuli are properly interpreted.

Maturation of several functionally distinct nociceptor subclasses also requires NGF-TrkA signaling during embryonic and postnatal nociceptor development (Luo et al., 2007; Gascon et al., 2010; Marmigère and Ernfors, 2007). The two major populations of mature nociceptor subclasses are peptidergic and nonpeptidergic nociceptors. Peptidergic nociceptors express TrkA, calcitonin gene-related peptide (CGRP), and substance P throughout development and are responsive to target-derived NGF (Lallemend and Ernfors, 2012). Nonpeptidergic nociceptors do not express TrkA or neuropeptides, but rather express an RTK, Ret, that is responsive to the target-derived glial cell line-derived neurotrophic factor (GDNF) family of ligands (Airaksinen and Saarma, 2002; Molliver et al., 1997). Loss of GDNF family receptor α2 (GFRα2), a Ret coreceptor that responds to the

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GDNF family ligand neurturin (NRTN), impairs peripheral target innervation and sensitivity to inflammatory pain (Lindfors et al., 2006). Interestingly, acute pain is sensed by TrkA+ peptidergic nociceptors in the absence of NRTN signaling, suggesting that TrkA+ and Ret+ nociceptors are functionally distinct from one another in adulthood despite being derived from the same early TrkA+ nociceptor lineage during embryonic development (Marmigère and Ernfors, 2007; Liu and Ma, 2011). Together, these studies suggest that NGF-TrkA signaling is critical for the orchestration of peptidergic and nonpeptidergic nociceptive circuits that underlie the perception of distinct forms of painful touch.

Given the broad phenotypes and functions that NGF-TrkA signaling regulates, it stands to reason that there should be factors that modulate TrkA signals and, by extension, nociceptor development and function. Clues for this hypothesis come from studies in the sympathetic nervous system, a system that also requires NGF-TrkA signaling for survival and target innervation (Crowley et al., 1994; Glebova and Ginty, 2004). In sympathetic neurons, antagonism of NGF-TrkA signals can occur via the p75 neurotrophin receptor (p75NTR) that induces death (Deppmann et al., 2008), promotes axon pruning (Singh et al., 2008), dampens excitability (Luther and Birren, 2009), and restricts postsynaptic densities (Sharma et al., 2010). As such, elimination of *p75NTR* is analogous to TrkA gain of function in sympathetic neurons. Consequently, one would expect that the loss of p75NTR in nociceptors would manifest as heightened pain sensitivity. However, numerous reports argue the opposite; loss of p75NTR results in decreased pain sensitivity and reduced cutaneous innervation by nociceptors (Lee et al., 1992; Bergmann et al., 1997). Nociceptors from *p75NTR*$^{-/-}$ mice are roughly 3-fold less sensitive to NGF (Davies et al., 1993; Lee et al., 1994), suggesting that p75NTR likely augments NGF-TrkA signaling in nociceptors.

If not p75NTR, might a different receptor gate NGF-TrkA signaling in nociceptors? It is possible that receptors structurally related to p75NTR may function as negative regulators of NGF-TrkA signals in nociceptors. Indeed, p75NTR is just one of over 25 tumor necrosis factor (TNF) receptor (TNFR) superfamily members, many of which are implicated in the modulation of growth factor signaling (Deppmann and Janes, 2013; Locksley et al., 2001). To test this idea, we screened for the expression of 23 TNFR family members in the dorsal root ganglia (DRG); this screening revealed the selective expression of three highly related TNFR family members: TNFR1, p75NTR, and death receptor 6 (DR6). Herein we pursued the role of TNFR1 signaling in nociceptor development in part because it has been shown to negatively regulate events such as neurite growth and survival in the sympathetic nervous system (Barker et al., 2001; Kisiswa et al., 2013). Similar to phenotypes observed in the sympathetic nervous system, we report that TNFR1 forward signaling can also inhibit the development of NGF-TrkA-dependent sensory circuits by negatively regulating neurite growth, cell number, and excitability of TrkA+ primary nociceptors. Moreover, loss of either TNF-α or TNFR1 forward signaling drives TrkA+ nociceptors toward a premature nonpeptidergic fate. Surprisingly, we found that TNFR1 plays a dichotomous role in the function of peptidergic and nonpeptidergic nociceptors. Although

TrkA+ peptidergic nociceptors exhibit increased responsiveness to NGF as evidenced by enhanced growth and excitability in the absence of TNFR1 or TNF-α, the Ret+/TrkA− population of nonpeptidergic nociceptors exhibits functional impairments in NRTN-dependent neurite outgrowth and excitability due to absence of TNFR1-TNF-α reverse signaling. We demonstrate that *Tnf* and *Tnfr1* null mice are hypersensitive to several pain modalities, likely caused by enhanced nociceptor sensitivity to NGF. Finally, we provide genetic and biochemical evidence that the increased gain in nociceptive signals observed in *Tnf* or *Tnfr1* knockouts is linked to an increase in the NGF sensitivity of TrkA+ nociceptors. These results suggest that TNF-α and TNFR1 coordinate the development and function of molecularly distinct nociceptive circuits through crosstalk with TrkA and Ret to either block or promote pain sensitivity in peptidergic and nonpeptidergic nociceptor populations, respectively.

RESULTS

Characterization of TNF-α and TNFR1 Expression on Nociceptors and Their Targets

In order to identify putative factors that can antagonize NGF-TrkA-dependent signaling in nociceptors, we examined the expression of 23 TNFR family members in embryonic day (E) 18.5 DRG, brain, or muscle via RT-PCR (Figure S1A available online). This analysis revealed expression of three highly related TNFR family members in the DRG: *p75NTR* (TNFRSF16), *Dr6* (TNFRSF21), and *Tnfr1* (TNFRSF1A). We focused on TNFR1, an understudied TNFR family member in the context of nociceptor development and function. Given that TNFR1 is robustly expressed in the DRG, which contains many different cell types, we next sought to determine whether it is expressed on nociceptors. To this end, we performed immunohistochemistry to examine the percentage of colocalization of TNFR1 with CGRP, peripherin, or TrkA at postnatal day (P)0 (Figures 1A–1C). CGRP and TrkA represent peptidergic nociceptors, whereas peripherin labels small-diameter unmyelinated nociceptors. In each case, TNFR1 colocalizes with nociceptive markers >99% of the time (Figure S2A). In addition, we found that TNFR1 appears to be broadly expressed in the DRG as it colocalizes with parvalbumin+ proprioceptive neurons (Figure S2C).

We performed the same analysis at P30 when most nociceptors are terminally specialized into distinct functional subclasses (Lallemend and Ernfors, 2012). In addition to CGRP, TrkA, and peripherin, we examined nonpeptidergic neurons by staining with the fluorescently conjugated lectin IB4. We observed similar levels of colocalization at P30 as at P0 (i.e., at least >99% of nociceptors are positive for TNFR1) (Figures 1D–1G; Figure S2B). We validated the TNFR1 antibody (Figure S1B) and found that TNFR1 is localized to both the cell bodies and axons of sensory neurons both in vivo (Figure S1C) and in vitro (Figure S1D). Together, these analyses suggest that TNFR1 is ubiquitously expressed by nociceptors across different stages of sensory circuit development, ranging from early to mature nociceptor populations.

We also sought to localize the sources of TNF-α in developing nociceptive circuits. After validating the TNF-α antibody (Figures
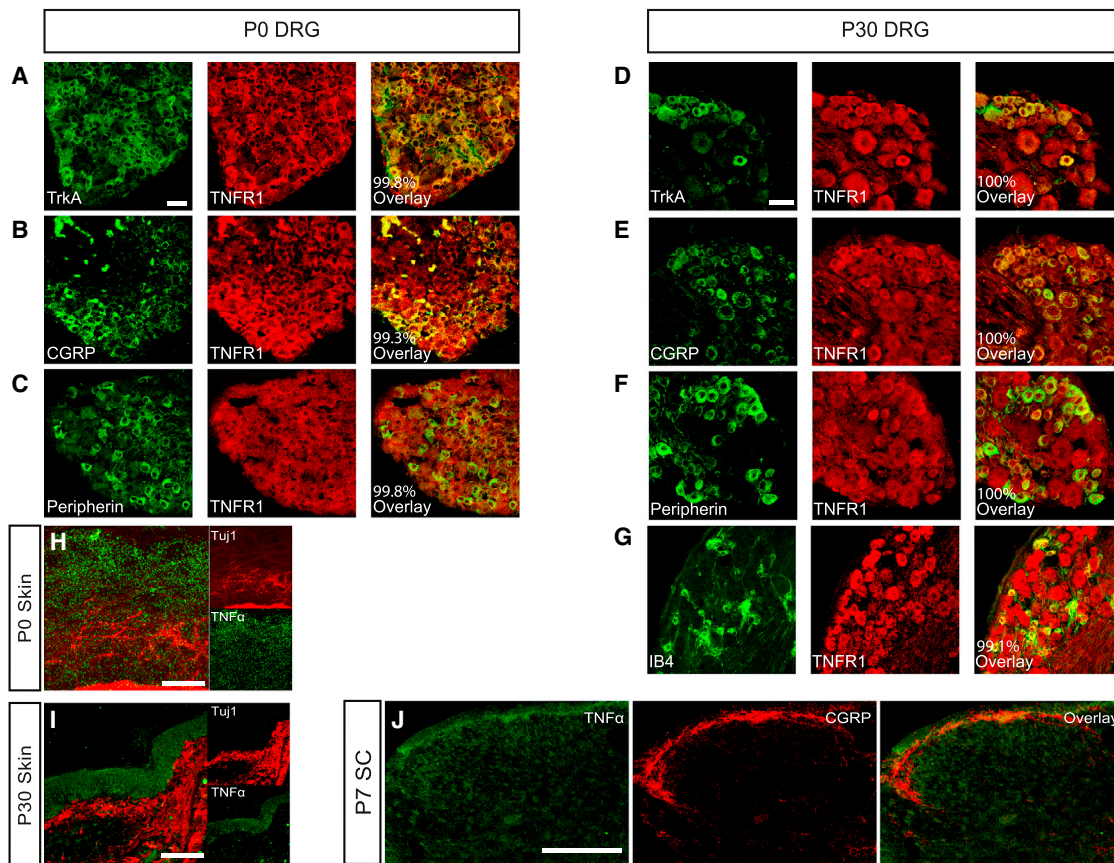
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Figure 1. TNFR1 Is Expressed by Nociceptive Neurons, and TNF-α Is Expressed in Nociceptor Targets
(A–C) Quantification of TNFR1 colocalization the nociceptive markers TrkA (A), CGRP (B), and peripherin (C) in the P0 DRG. Scale bar represents 30 μm.
(D–G) Quantification of TNFR1 colocalization with the nociceptive markers TrkA (D), CGRP (E), and peripherin (F) plus IB4 (G) as a marker of nonpeptidergic nociceptors in the P30 DRG. Scale bar represents 60 μm.
(H and I) TNF-α is enriched mainly in the epidermis at P0 (H) and P30 (I). Scale bar represents 120 μm.
(J) Expression of TNF-α in the marginal zone and dorsal horn neurons of the spinal cord. Scale bar represents 150 μm.
See also Figures S1 and S2.

S1E–S1G), we found that TNF-α is enriched on sensory neurons in the P4 DRG (Figure S1H), in the epidermis between P0 and P30 (Figures 1H and 1I), and in the marginal zone and second-order spinal cord neurons at P7 (Figure 1J). Importantly, we found that TNFR2, a close relative of TNFR1 that also binds TNF-α, was absent from the DRG (Figure S1A) and spinal cord (Figure S1I). Taken together these data suggest that sensory neurons, peripheral targets, and central targets express TNF-α, which may signal to nociceptors exclusively through TNFR1 during critical periods of nociceptor circuit development and maturation.

TNF-α-TNFR1 Signaling Suppresses Skin Innervation and NGF-Dependent Neurite Growth Programs

During normal developmental processes, a critical function of NGF-TrkA signaling is control of nociceptor neurite growth into peripheral targets (Patel et al., 2000). If TNF-α-TNFR1 signaling antagonizes NGF-TrkA-dependent growth of nociceptors into peripheral targets, we would predict that axon overgrowth into the skin would be observed in $Tnfr1^{-/-}$ and $Tnf^{-/-}$ mice. To test whether TNF-α-TNFR1 signaling is indeed required, in vivo,

for proper axon innervation of peripheral targets, we performed immunohistochemical analyses for nociceptive fibers in the epidermis, as described previously (Zylka et al., 2005). First, we observed that there is an increased density of small-diameter peripherin+ fibers in glabrous skin at P0 in $Tnfr1^{-/-}$ and $Tnf^{-/-}$ animals relative to wild-type (WT) (Figure 2A). We then stained for the pan-axonal marker protein gene product 9.5 (PGP9.5) and observed twice as many PGP9.5+ nociceptive fibers projecting into the epidermis in $Tnfr1^{-/-}$ or $Tnf^{-/-}$ mice than WT mice by P30, suggesting that TNF-α and TNFR1 normally suppress neurite growth programs in the periphery (Figures 2B and 2C). Finally, whole-mount substance P immunostaining of the forepaw revealed that as early as E14.5, nociceptive fibers densely innervate the digits and footpad of the paw in mice lacking TNF-α or TNFR1 (Figure 2D). Taken together these data suggest that the TNF-α-TNFR1 pathway is critical in regulating growth and refinement of nociceptor peripheral projections in vivo.

To examine the role of TNFR1 on NGF-TrkA-dependent neurite growth in vitro, we established dissociated sensory neurons from WT, $Tnfr1^{-/-}$, or $Tnf^{-/-}$ mice in microfluidic devices, which
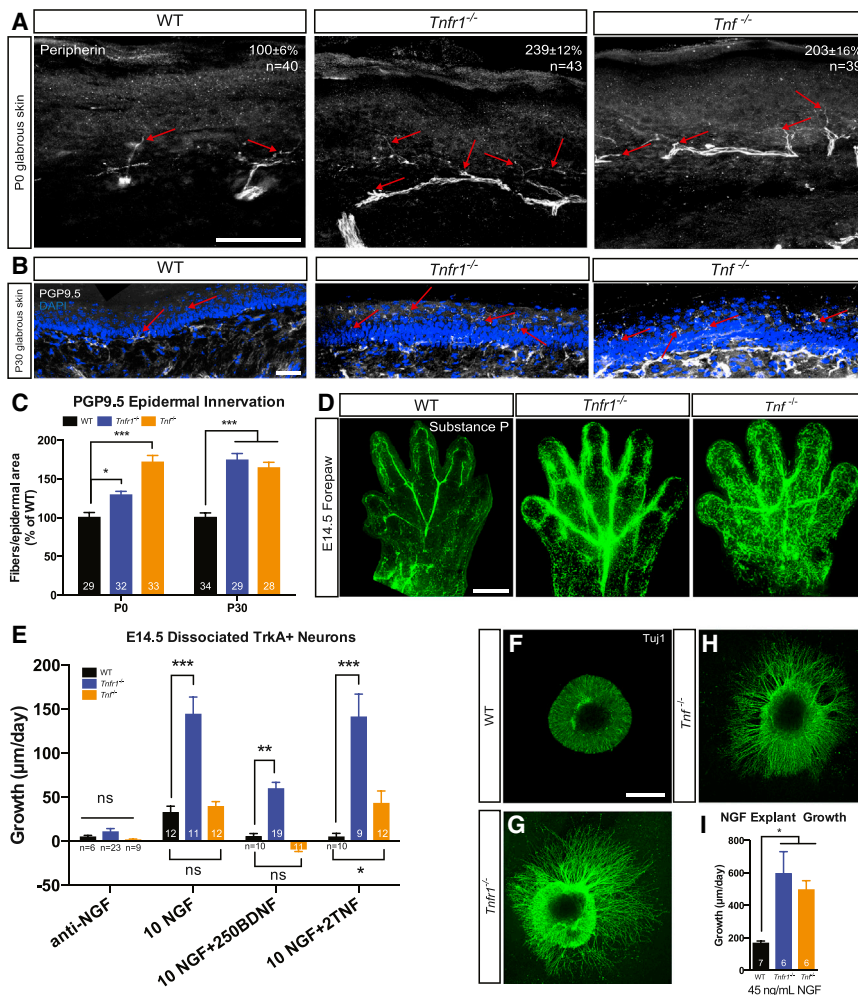
Figure 2. *Tnfr1*−/− and *Tnf*−/− Nociceptive Fibers Hyperinnervate the Skin and Are Hypersensitive to NGF-Dependent Growth

(A) Peripherin immunostaining of hindpaw thin glabrous skin. Arrows point to axons sprouting into the cutaneous field. Scale bar represents 50 μm. Quantification shown is peripherin+ fiber cutaneous field density normalized as percent of WT mean. Five animals of each genotype were examined.

(B and C) PGP9.5 immunostaining of thin glabrous skin and quantification of epidermal innervation. (B) P30 thin glabrous skin. Arrows point to invading fibers. (C) Quantification of the number of PGP9.5 neurites crossing into the epidermal field at P0 and P30, normalized to percent of WT. Five animals of each genotype were analyzed per time point. Scale bar represents 25 μm.

(D) Representative whole-mount immunostaining of E14.5 forepaws for the peptidergic nociceptor marker substance P; n = 4 paws from four mice stained per genotype. Scale bar represents 50 μm.

(E) In vitro neurite growth of E14.5 TrkA+ nociceptors grown in NGF and measured in compartmentalized chambers. Data shown are from two to six independent experiments for each condition.

(F–I) Tuj1 immunostaining in WT (F), *Tnfr1*−/− (G), and *Tnf*−/− (H) and quantification (I) of E14.5 explant outgrowth in response to 45 ng/ml NGF for 24 hr. Scale bar represents 500 μm. Explants were from three or more mice per genotype.

One-way (I) or two-way (C and E) ANOVA, Bonferroni posttest. Data represent mean ± SEM. ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001.

spatially isolate neuronal cell bodies and neurites (Sharma et al., 2010). We found that WT neurons bathed in NGF along with TNF-α or brain-derived neurotrophic factor (BDNF) showed significantly lower rates of neurite growth than WT neurons bathed in NGF alone (Figure 2E). Strikingly, *Tnfr1*−/− neurites grew roughly five times faster than WT neurons in response to 10 ng/ml NGF, suggesting that TNFR1 plays a role in suppressing NGF-dependent neurite growth (Figure 2E). Although TNF-α had no effect on NGF-dependent neurite growth in *Tnfr1*−/− neurons, we observed that BDNF still slowed neurite growth in *Tnfr1*−/− neurons bathed in NGF, albeit to a lesser degree than BDNF treatment on WT mice neurons (Figure 2E). Interestingly, neurite growth of dissociated *Tnf*−/− neurons revealed separable roles for TNF-α and TNFR1, suggesting that TNFR1 may suppress nociceptor neurite growth in the absence of TNF-α. To assess the effect of NGF-mediated neurite growth on whole ganglia from *Tnfr1*−/− and *Tnf*−/− mice, E14.5 DRG explants were cultured in NGF, yielding results corroborating excessive NGF-dependent growth in the *Tnfr1*−/− and *Tnf*−/− explants; *Tnfr1*−/− and *Tnf*−/− neurites grew approximately three times faster than WT neurites (Figures 2F–2I). It is possible that paracrine, rather than autocrine, axon pruning programs are more dominant,

yielding the difference in relative neurite growth rates between dissociated *Tnf*−/− nociceptors and explant cultures. Together, these data suggest that neurons lacking *Tnfr1* or *Tnf* are more responsive to NGF in the context of in vitro neurite growth assays due to loss of TNF-α-TNFR1 forward signaling.

TNF-α-TNFR1 Signaling Is Required for Proper Guidance of Peptidergic Central Projections

In *Tnfr1*−/− and *Tnf*−/− mice, nociceptor peripheral projections display phenotypes reminiscent of those observed in classical NGF-TrkA gain-of-function experiments, demonstrating fiber overgrowth into peripheral targets (Aloe et al., 1975). Therefore, we sought to test whether nociceptor central projections were disrupted similar to peripheral projections in *Tnfr1*−/− and *Tnf*−/− mice. To address this, we compared peptidergic (TrkA+, CGRP+) central projections in the spinal cord between WT, *Tnfr1*−/−, and *Tnf*−/− mice at P0, P7, and P30. We noted complete segregation of peptidergic (CGRP+) and nonpeptidergic (IB4+) axons in the dorsal horn by P30 (Figures S3A–S3C), suggesting that nonpeptidergic nociceptor fate specification is intact (Chen et al., 2006b). However, we found that mice lacking *Tnfr1* or *Tnf* display an ∼50% larger area occupied by TrkA+ and CGRP+ peptidergic fibers than WT lumbar dorsal horn at P0 (Figures 3A–3G). Moreover, most sections contained axon bundles
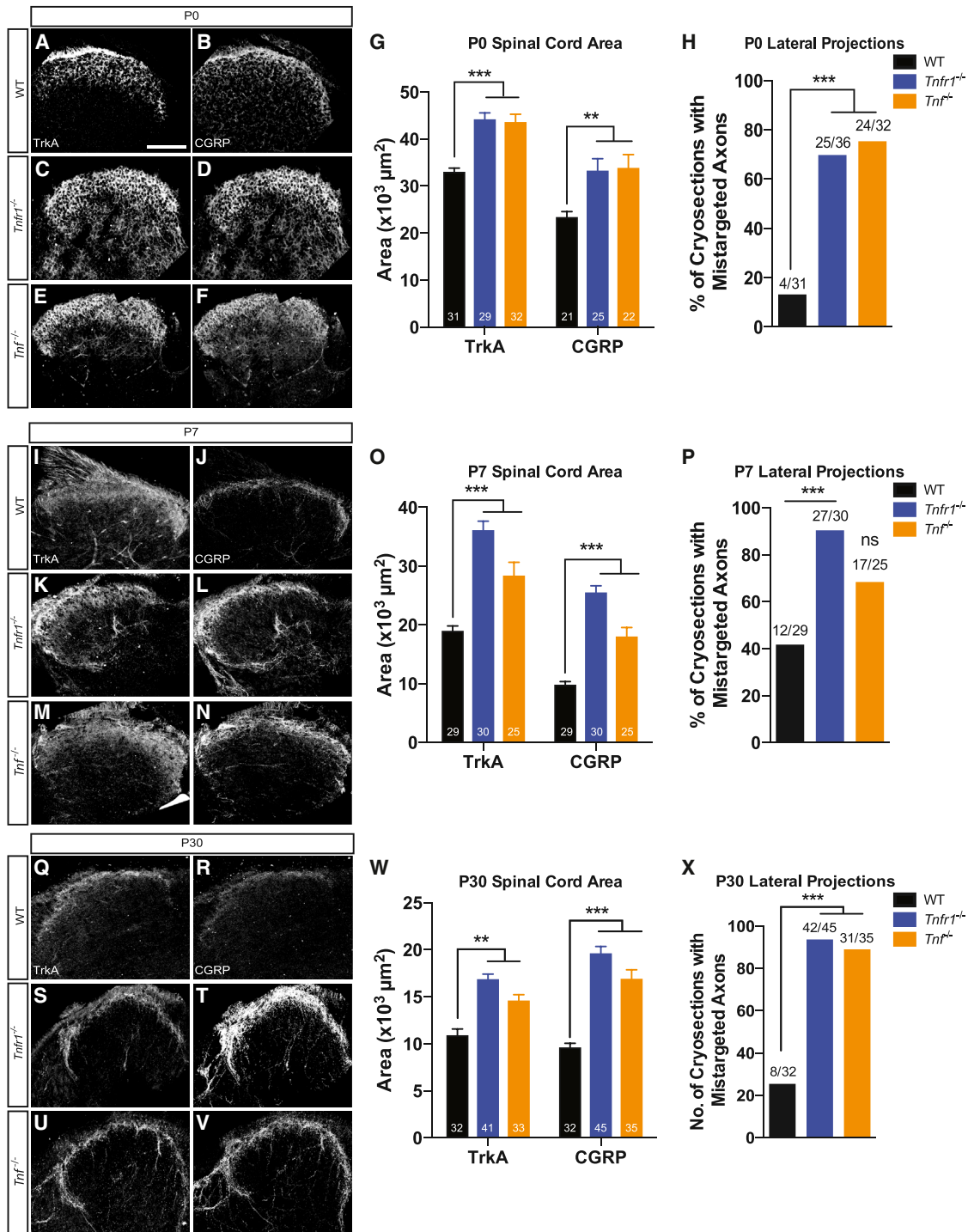
Figure 3. *Tnfr1*^{−/−} and *Tnf*^{−/−} Peptidergic Central Projections Are Robust and Misguided

(A–H) TrkA and CGRP immunostaining in P0 WT (A and B), *Tnfr1*^{−/−} (C and D), and *Tnf*^{−/−} (E and F) lumbar spinal cord. Quantification of area (G) and lateral projections (H) within WT, *Tnfr1*^{−/−}, and *Tnf*^{−/−} spinal cord sections. Scale bar represents 100 μm.

(I–X) Immunostaining and quantification of P7 (I–P) and P30 (Q–X) lumbar spinal cord.

See also Figure S3. Two-way ANOVA, Bonferroni posttest (G, O, and W) and Fisher's exact test (H, P, and X). Five mice were analyzed per genotype per time point. Data represent mean ± SEM. ns, not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
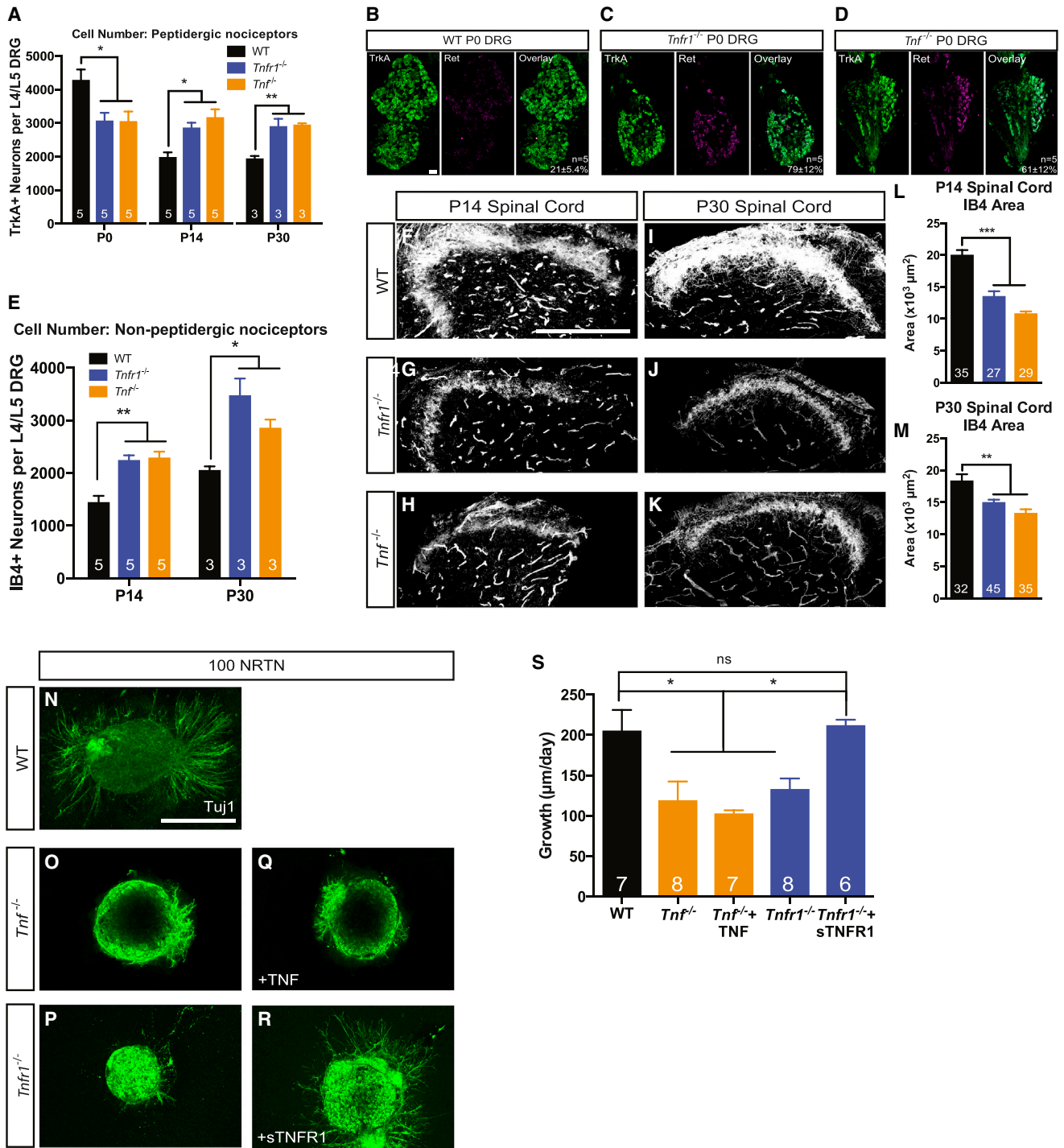
Figure 4. Deletion of *Tnfr1* or *Tnf* Leads to the Premature Differentiation and Impaired Growth of Nonpeptidergic Nociceptors

(A) Quantification of the number of TrkA+ sensory neurons in the L4/L5 DRG at P0, P14, and P30.

(B–D) Representative images of Ret/TrkA colocalization in the P0 DRG from WT (B), *Tnfr1*^{−/−} (C), and *Tnf*^{−/−} (D) mice. Scale bar represents 30 μm. Quantification of colocalization is shown on overlay.

(E) Quantification of the number of IB4+ neurons in the L4/L5 DRG at P14 and P30.

(F–M) Immunostaining and quantification of IB4+ axons invading the spinal cord dorsal horn at P14 (F–H) and P30 (I–K) in WT, *Tnfr1*^{−/−}, and *Tnf*^{−/−} mice. Quantification of IB4+ axon area shown in (L) and (M). Scale bar represents 100 μm.

(legend continued on next page)

that misprojected within the dorsal horn and extended medially and/or laterally (Figure 3H). The same analyses performed at P7 and P30 revealed similar phenotypes, suggesting that central projection defects are stable even after terminal differentiation (Figures 3I–3X). The enhanced growth and misguidance of $Tnfr1^{-/-}$ and $Tnf^{-/-}$ central projections are reminiscent of other pathways that negatively modulate NGF-dependent central projections such as HoxD1 (Guo et al., 2011), suggesting that TNF-α-TNFR1 signaling is also required for proper growth and refinement of nociceptor central projections.

TNF-α-TNFR1 Signaling Coordinates Nociceptor Differentiation

There are several possible explanations for the increase in peptidergic peripheral and central projection densities that occur in $Tnfr1^{-/-}$ and $Tnf^{-/-}$ mice: (1) an increase in the neurite density of individual nociceptors; and/or (2) more nociceptors in the DRG, which would increase the overall number of fibers projecting to the skin and spinal cord. Although we have established that loss of TNFR1 signaling enhances NGF-dependent neurite growth, it is also possible that TNFR1 antagonizes nonaxon growth aspects of NGF-TrkA signaling such as cell death and differentiation in the DRG. To test this possibility we examined cell number in the DRG by counting the number of TrkA+ nociceptors at P0, P14, and P30, time points corresponding to key regulatory events in the survival and differentiation of nociceptors (Marmigère and Ernfors, 2007). Surprisingly, we observed a significant decrease in the TrkA+ nociceptor population at P0 in $Tnfr1^{-/-}$ and $Tnf^{-/-}$ mice but, relative to WT, more TrkA+ neurons at P14 and P30 (Figure 4A). It is possible that the appearance of fewer TrkA+ neurons at P0 may not be due to an altered trophic threshold for survival; rather, it may have to do with altered differentiation programs. Indeed, it is established that early TrkA+ nociceptors downregulate TrkA/CGRP and upregulate Ret to become nonpeptidergic nociceptors through a process that is itself dependent on the strength of NGF-TrkA signals (Luo et al., 2007). Thus, if a Tnf or $Tnfr1$ deletion enhances NGF sensitivity in TrkA+ nociceptors, an increased number of early TrkA+ neurons would be driven toward a nonpeptidergic fate. To examine this possibility, we assessed putative nonpeptidergic cell number in the DRG by counting the number of Ret+/TrkA+ (differentiating) and IB4+ (terminally differentiated) neurons across time, as described previously (Luo et al., 2007). The number of differentiating Ret+/TrkA+ cells in $Tnfr1^{-/-}$ or $Tnf^{-/-}$ P0 DRGs was approximately three times higher than the number of differentiating neurons in WT mice (Figures 4B–4D). At P14 and P30, the increase in nonpeptidergic neuron number persists in $Tnfr1^{-/-}$ and $Tnf^{-/-}$ mice compared to WT (Figure 4E). These data suggest that TNF-α-TNFR1 signals work during nociceptive differentiation to dampen the NGF-TrkA-dependent drive toward a nonpeptidergic fate and that loss of these signals leads to higher numbers of both peptidergic and nonpeptidergic nociceptors.

TNFR1-TNF-α Signaling Is Required for Nonpeptidergic Nociceptor Axon Extension into the Spinal Cord and NRTN-Dependent Neurite Outgrowth

We next sought to determine whether TNF-α and TNFR1 specifically influence developmental processes in TrkA+ neurons or can more broadly affect other nociceptor pools such as Ret-expressing nonpeptidergic neurons. To address this we used WT, $Tnfr1^{-/-}$, and $Tnf^{-/-}$ mice to evaluate whether the extent of nonpeptidergic central projections would reflect the robust expansion into the dorsal horn observed for peptidergic nociceptor central projections. Surprisingly, there was approximately a 30%–50% reduction in the area devoted to nonpeptidergic, IB4+ axons in the dorsal horn at P14 and P30 in $Tnfr1^{-/-}$ or $Tnf^{-/-}$ mice relative to WT mice (Figures 4F–4M). Having observed an increase in the number of these nonpeptidergic, IB4+ cells in $Tnfr1^{-/-}$ and $Tnf^{-/-}$ mice, this finding suggests that TNF-α-TNFR1 signaling is required for the maintenance of nonpeptidergic nociceptor central projections.

A well-established signaling pathway implicated in promoting the growth of nonpeptidergic nociceptive axons is through the ligand, coreceptor, and RTK: NRTN, GFRα2, and Ret, respectively (Stucky and Lewin, 1999; Airaksinen and Saarma, 2002; Lindfors et al., 2006; Luo et al., 2007). Therefore, we next asked whether loss of TNF-α or its receptor could influence Ret-dependent neurite growth. To this end, we cultured E14.5 DRG explants from WT, $Tnfr1^{-/-}$, and $Tnf^{-/-}$ mice in 100 ng/ml NRTN. Surprisingly, in response to NRTN, neurite outgrowth was significantly lower in $Tnfr1^{-/-}$ and $Tnf^{-/-}$ explants compared to WT (Figures 4N–4P), consistent with in vivo observations of decreased density of nonpeptidergic axons in lamina IIi of the spinal cord. We initially attempted to rescue this impaired neurite outgrowth by activating TNF-α-TNFR1 forward signaling through incubation of $Tnf^{-/-}$ explants in TNF-α+NRTN (Figure 4Q), which yielded no significant differences compared to the growth of $Tnf^{-/-}$ explants grown solely in NRTN (Figure 4S). Given that a recent study implicated TNFR1-TNF-α reverse signaling (Sun and Fink, 2007) in the growth of sympathetic axons (Kisiswa et al., 2013), we hypothesized that perhaps TNF-α-TNFR1 reverse signaling, rather than forward signaling, is responsible for nonpeptidergic neurite outgrowth. We tested this idea by incubating E14.5 explant cultures in NRTN as well as selective readdition of soluble TNFR1 (sTNFR1) to $Tnfr1^{-/-}$ explants to activate TNFR1-TNF-α reverse signaling pathways. Consistent with a reverse signaling paradigm, sTNFR1 addition to $Tnfr1^{-/-}$ explants restored neurite growth to WT levels (Figures 4R and 4S). These data suggest that in contrast to the repressive effects of TNF-α-TNFR1 forward signaling on NGF-TrkA-dependent growth, a TNFR1-TNF-α reverse signaling program enhances NRTN-GFRα2-Ret-dependent neurite growth in nonpeptidergic nociceptors.

(N–S) E14.5 DRG explants cultured in 100 ng/ml NRTN (N–P), 100 ng/ml NRTN + 2 ng/ml TNF (Q), or 100 ng/ml NRTN + 5 μg/ml sTNFR1 (R) for 24 hr. Explants are from three or more mice per condition. Scale bar represents 100 μm.

One-way ANOVA, Bonferroni posttest (A, L, and M) or Tukey posttest (S). Two-way ANOVA, Bonferroni posttest (E). Number of mice analyzed is indicated in (A) and (E). Five mice were analyzed in (L) and (M). Data represent mean ± SEM. ns, not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
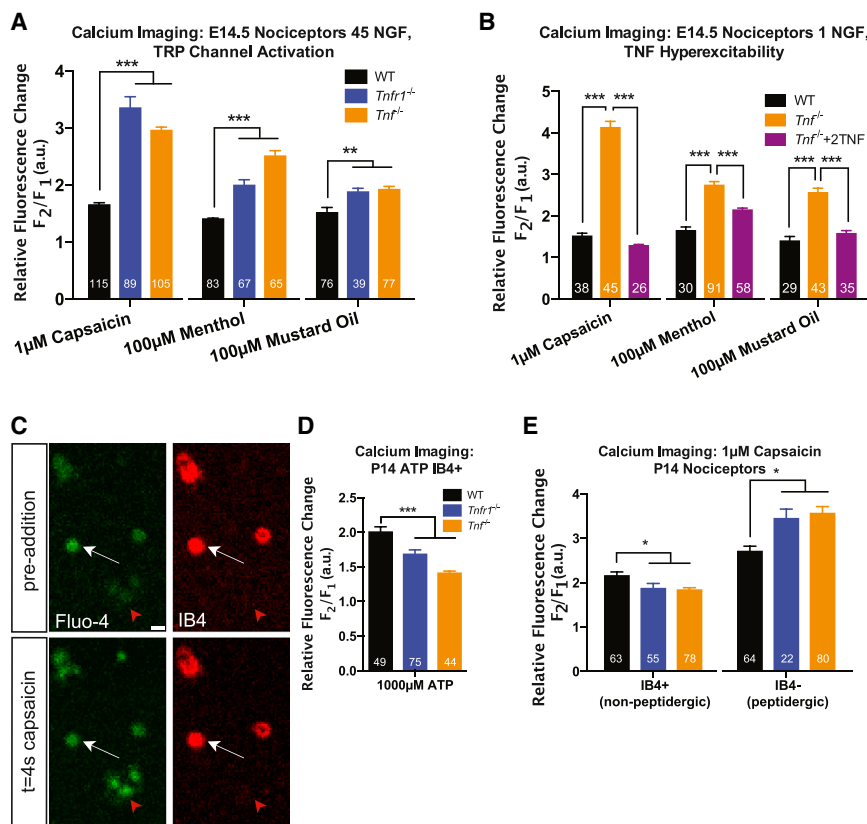
Figure 5. TNF-α-TNFR1 Signaling Differentially Regulates the Excitability of Peptidergic and Nonpeptidergic Nociceptors

(A) Fluo-4 AM in vitro calcium imaging of E14.5 nociceptors. The changes in fluorescence of E14.5 TrkA+ nociceptors were measured (cultured in 45 ng/ml NGF for 3 days in vitro) after acute addition of the indicated TRP channel agonist. Two experiments per condition are reported; at least three mice per experiment were used.

(B) Rescue experiment demonstrating that TNF-α is sufficient to quench the hyperexcitability of $Tnf^{-/-}$ nociceptors. E14.5 nociceptors from WT or $Tnf^{-/-}$ mice were cultured in 1 ng/ml NGF (with or without TNF for $Tnf^{-/-}$ neurons) for 2 days in vitro and prepared for calcium imaging as in (A). Two experiments per condition are reported; at least three mice per experiment were used.

(C) Representative image of P14 calcium imaging strategy examining nonpeptidergic nociceptor excitability. IB4+ and IB4– neurons are shown before and after agonist addition. Red arrow, IB4– neuron; white arrow, IB4+ neuron. Scale bar represents 20 μm.

(D) Quantification of fluorescence changes of P14 nociceptors (cultured in 45 ng/ml NGF for 1 day in vitro) in response to 1,000 μM ATP.

(E) Excitability of P14 IB4+ and IB4– nociceptors responding to capsaicin. Two to three experiments per condition are reported; three mice per experiment were used.

See also Figure S4. Statistics determined by one-way (A, B, D, and E) ANOVA, Bonferroni posttest. Data represent mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

TNF-α and TNFR1 Are Required to Differentially Regulate the Excitability of Peptidergic and Nonpeptidergic Nociceptors

Nociceptors sense thermal, mechanical, and chemical stimuli through the expression of different ion channels such as the transient receptor potential (TRP) family of ion channels (Clapham, 2003; Moran et al., 2011). Importantly, RTK signaling modulates the excitability of ion channels in nociceptors. For instance, NGF-TrkA signaling can enhance the expression and sensitivity of TRP ion channels in peptidergic nociceptors (Chuang et al., 2001; Zhang et al., 2005; Ji et al., 2002; Luo et al., 2007), whereas NRTN-GFRα2-Ret signaling regulates expression of the ATP-gated ion channel P2X purinoceptor 3 in nonpeptidergic nociceptors (Wang et al., 2013). To test whether $Tnfr1^{-/-}$ and $Tnf^{-/-}$ peptidergic nociceptors exhibit altered excitability, we performed calcium imaging in cultured E14.5 TrkA+ sensory neurons, similar to previous studies (Liu et al., 2009). Neurons were stimulated with mustard oil, menthol, and capsaicin, specific agonists of the temperature-sensitive TRP channels TRPA1, TRPM8, and TRPV1, respectively (Caterina et al., 1997; Peier et al., 2002; Bandell et al., 2004). Upon stimulation with any of the three agonists, $Tnfr1^{-/-}$ and $Tnf^{-/-}$ TrkA+ neurons were hypersensitive to each of the chemical analogs relative to WT neurons (Figure 5A). We also conducted a rescue experiment to determine whether TNF-α is sufficient to quench the hyperexcitability of $Tnf^{-/-}$ nociceptors. We incubated

E14.5 nociceptors from WT and $Tnf^{-/-}$ mice in 1 ng/ml NGF and observed hyperexcitability of $Tnf^{-/-}$ nociceptors relative to WT, analogous to the effects observed in 45 ng/ml NGF. However, when we selectively added 2 ng/ml TNF-α to $Tnf^{-/-}$ nociceptors cultured in 1 ng/ml NGF, we were able to restore TRP channel activity to WT levels (Figure 5B). This suggests that TNF-α-TNFR1 forward signaling negatively regulates the excitability of TRP channels in TrkA+ nociceptors and most prominently, TRPV1. In addition, the reported fluorescence fold changes are somewhat less robust than those reported in the literature due to a longer working distance in the z-plane during live imaging sessions (Figures S4A–S4C). This did not influence our ability to accurately assess relative excitability between genotypes (Figure S4D).

We also tested whether the excitability of nonpeptidergic nociceptors is altered in the absence of TNF-α or TNFR1 because NRTN is known to regulate sensitivity of P2X ion channels (Wang et al., 2013). We used a strategy that permits recording fluorescence changes exclusively in nonpeptidergic nociceptors by incubating the cultures with Alexa 568-conjugated IB4 before application of a chemical agonist (Figure 5C) (Gerevich et al., 2004). In response to ATP stimulation, $Tnfr1^{-/-}$ and $Tnf^{-/-}$ P14 IB4+ nociceptors were less excitable than WT IB4+ neurons (Figure 5D). Because nonpeptidergic nociceptors also express TRPV1 (Chen et al., 2006b; Luo et al., 2007), we tested whether a similar effect was observed after capsaicin
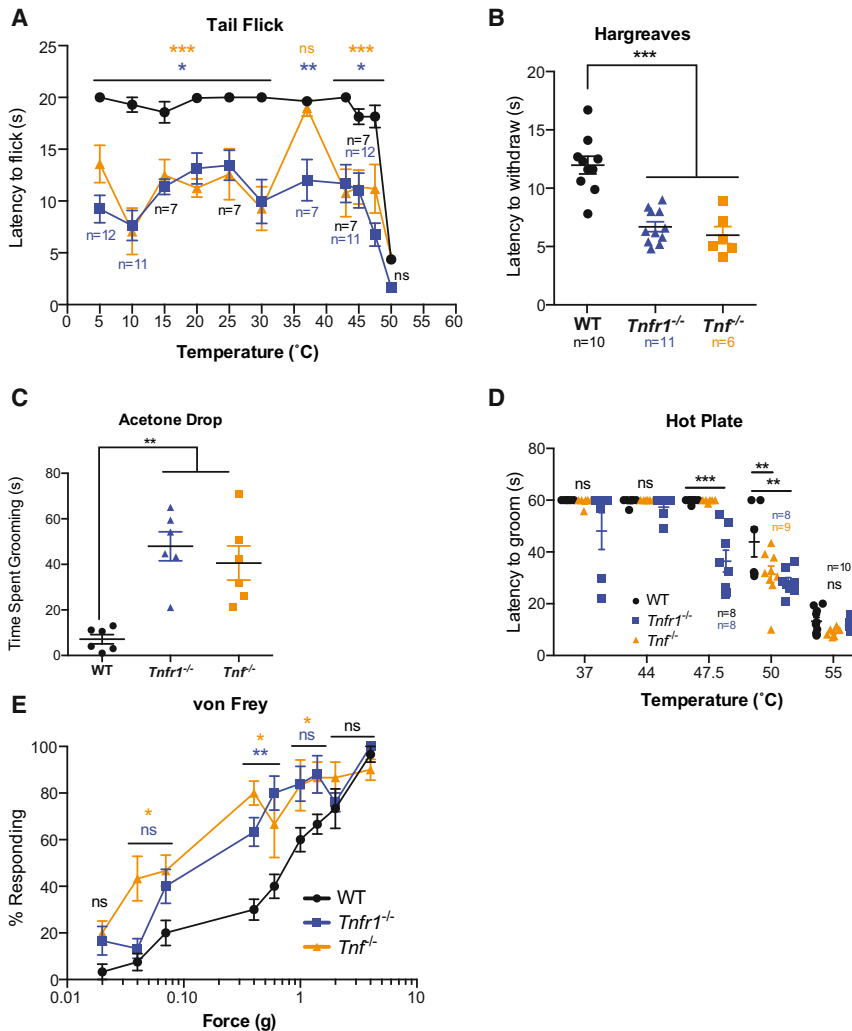
Figure 6. *Tnfr1*⁻/⁻ and *Tnf*⁻/⁻ Mice Are Hypersensitive to Thermal and Mechanical Stimuli

(A–E) Behavioral sensitivity of WT, *Tnfr1*⁻/⁻, and *Tnf*⁻/⁻ mice in response to the tail flick (A), Hargreaves radiant heat (B), acetone drop (C), hot plate (D), and von Frey (E) tests measuring reflexive (A and B) or centrally mediated (C and D) thermal pain thresholds or mechanical acuity (E).

We used n = 6 mice per data point (aged P26–P56); if n > 6 mice, it is indicated. Two-way (A, D, and E) or one-way (B and C) ANOVA, Bonferroni posttest. All mice shown are on a B6;129s mixed background. WT mice are background matched, nonlittermate controls for *Tnfr1*⁻/⁻ and *Tnf*⁻/⁻ mice. See also Figure S5. Data represent mean ± SEM. ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001.

stimulation. Indeed, IB4+ neurons from *Tnfr1*⁻/⁻ or *Tnf*⁻/⁻ nociceptors were hypoexcitable to capsaicin stimulation compared to WT, whereas IB4− nociceptors were hyperexcitable (Figure 5E). As observed for axon growth, these data suggest opposing regulatory roles for TNF-α and TNFR1 in attenuating excitability of TrkA+ peptidergic nociceptors or enhancing TRP channel activity of Ret+ nonpeptidergic nociceptors.

Tnf⁻/⁻ and *Tnfr1*⁻/⁻ Mice Are Hypersensitive to Thermal and Mechanical Stimuli

NGF-TrkA signaling controls nociceptor target innervation, cell survival, fate specification, excitability, and as a result, pain sensitivity thresholds (Pezet and McMahon, 2006). Overactive NGF signaling therefore causes hyperalgesia largely through pathological hyperactivation of pathways regulating target innervation and excitability (Smelter and Hochberg, 2013; Hefti et al., 2006; Pezet and McMahon, 2006). We found that excessive NGF signaling and loss of TNFR1 or TNF-α signaling phenocopy one another with respect to hyperinnervation of the skin and spinal cord (Figures 2 and 3), hypersensitivity to TRP channel agonists (Figure 5), as well as a greater overall number of nociceptors (Fig-

ures 4A and 4E). Thus, we hypothesized that developmental loss of TNF-α-TNFR1 signaling would translate to increased sensitivity to pain analogous to injection with NGF (Lewin et al., 1993), which can be assessed by behavioral assays (for review, see Sandkühler, 2009). To test this hypothesis, we first sought to precisely corroborate our calcium imaging data by subjecting the mice to a variety of different temperatures at prescribed intervals. Toward this end, we performed the tail flick assay where tails of WT or mutant mice were submerged in water ranging from 5°C to 50°C for no more than 20 s, and the latency to tail withdrawal was measured. *Tnfr1*⁻/⁻ and *Tnf*⁻/⁻ mice were significantly more sensitive to every temperature examined, including neutral temperatures between 25°C and 37°C (Figure 6A). Hyperexcitability to all temperatures is consistent with the sensitization of TRPV1, TRPM8, and TRPA1 observed in our calcium imaging experiments (Figure 5A) because *Tnfr1*⁻/⁻ and *Tnf*⁻/⁻ mice were more responsive to hot, cool, and freezing temperatures, respectively, in the behavioral assays. *Tnfr1*⁻/⁻ and *Tnf*⁻/⁻ mice also responded more quickly than WT in the Hargreaves radiant heat test, which applies heat with no tactile cue (Figure 6B), further corroborating hypersensitivity to heat in the absence of TNF-α or TNFR1. These data are consistent with the notion that TNF-α-TNFR1 forward signaling contributes to proper perception of temperature by reducing nociceptor sensitivity thresholds.

In addition to measuring reflexive pain, we used two tests that interrogate centrally mediated hot- and cold-sensing abilities: the hot plate test and the acetone drop test. These tests quantify grooming responses indicative of which temperatures mice perceive as noxious. For the hot plate test we examined several temperatures ranging from mild to noxious heat. Both ligand and receptor null mice also displayed similar increases in grooming behaviors in the acetone drop test, which simulates cold

sensation (Figure 6C). Mice lacking *Tnfr1* and *Tnf* had lower pain thresholds for heat and consequently, an altered perception of thermal stimuli compared to WT mice (Figure 6D). In addition to enhanced reflexive acuity to pain, *Tnfr1*$^{-/-}$ and *Tnf*$^{-/-}$ mice display skewed thresholds of which temperatures are noxious and which are not, as they confuse the distinction between mild and injurious temperatures in both tests.

To assay another form of tactile sensitivity, we tested mechanical acuity in *Tnfr1*$^{-/-}$ and *Tnf*$^{-/-}$ mice. Reflexive mechanical sensitivity can be probed with von Frey filaments of varying diameter to determine the threshold sensitivity of a mechanical force. In response to mild mechanical forces, both *Tnfr1*$^{-/-}$ and *Tnf*$^{-/-}$ mice were more acutely sensitive to von Frey filament stimulation than WT (Figure 6F), suggesting that TNF-α-TNFR1 signaling can also shape and suppress perception of mechanical force in addition to reflexively and centrally mediated thermal pain. These data were independently confirmed using littermate controls 6–7 weeks of age (Figures S5A–S5E). Thus, we propose that the TNF-α-TNFR1 forward signaling pathway functions to tune primary nociceptors to properly interpret polymodal tactile stimuli.

Deletion of *Tnfr1* Rescues Pain Phenotypes Associated with *Ngf* Heterozygosity

Our in vitro and in vivo data demonstrate a consistent role for TNF-α-TNFR1 antagonism of NGF-TrkA function. Based on these data we speculated that by modulating one pathway we might be able to correct phenotypes observed in the other. Although *Ngf*$^{-/-}$ mice die perinatally (Crowley et al., 1994), it is established that nociceptors in mice heterozygous for *Ngf* have roughly 50% less cell survival and target innervation (Crowley et al., 1994; Brennan et al., 1999). If the role of TNF-α-TNFR1 signaling is to antagonize NGF-TrkA signaling in nociceptors, then we would predict that ablation of *Tnfr1* would rescue pain insensitivity phenotypes that might be associated with loss of one *Ngf* allele. First, we performed an analysis of the number of TrkA+ and IB4+ nociceptors in the P14 L4 or L5 DRG of *Tnfr1*$^{+/-}$, *Ngf*$^{+/-}$, *Tnfr1*$^{+/-}$;*Ngf*$^{+/-}$, and *Tnfr1*$^{-/-}$;*Ngf*$^{+/-}$ mice. Consistent with the loss of 50% of target-derived NGF, *Ngf*$^{+/-}$ and *Tnfr1*$^{+/-}$;*Ngf*$^{+/-}$ mice exhibited roughly 50% fewer TrkA+ and IB4+ neurons at P14 compared to *Tnfr1*$^{+/-}$ mice (Figure 7A). *Tnfr1*$^{-/-}$;*Ngf*$^{+/-}$ mice, by contrast, exhibited TrkA+ and IB4+ neuron numbers similar to *Tnfr1*$^{+/-}$, which suggests that loss of *Tnfr1* increases the NGF sensitivity of TrkA+ nociceptors, thereby rescuing the impaired cell survival caused by *Ngf* heterozygosity.

Next, we analyzed the central projections into the spinal cord dorsal horn in *Tnfr1*$^{+/-}$, *Ngf*$^{+/-}$, *Tnfr1*$^{+/-}$;*Ngf*$^{+/-}$, and *Tnfr1*$^{-/-}$;*Ngf*$^{+/-}$ mice. As expected (Crowley et al., 1994), there was roughly a 50% decrease in the area occupied by CGRP+ fibers in the dorsal horn at P14 in *Ngf*$^{+/-}$ and *Tnfr1*$^{+/-}$;*Ngf*$^{+/-}$ mice relative to *Tnfr1*$^{+/-}$ mice (Figures 7B–7F). Homozygous *Tnfr1* deletion can rescue this fiber deficiency in *Tnfr1*$^{-/-}$;*Ngf*$^{+/-}$ mice, providing additional evidence for functional antagonism between TNFR1 and TrkA in vivo. Although the number of nonpeptidergic nociceptors in the DRG is rescued by homozygous elimination of *Tnfr1*, the area occupied by nonpeptidergic axons in the dorsal horn was identical between *Ngf*$^{+/-}$, *Tnfr1*$^{+/-}$;*Ngf*$^{+/-}$,

and *Tnfr1*$^{-/-}$;*Ngf*$^{+/-}$ mice (Figures 7B and 7G–7J). This is consistent with the idea that TNFR1 positively regulates the maintenance of IB4+ fibers and is also consistent with the notion that TNFR1-TNF-α reverse signaling might be required for the full elaboration of nonpeptidergic axons through cooperation with NRTN signaling.

We next examined innervation of glabrous skin from the hindpaw footpad of P14 *Tnfr1*$^{+/-}$, *Ngf*$^{+/-}$, *Tnfr1*$^{+/-}$;*Ngf*$^{+/-}$, and *Tnfr1*$^{-/-}$;*Ngf*$^{+/-}$ mice by quantifying the number of CGRP+ fibers projecting into the epidermis (Figures 7K–7O). This analysis revealed a 50% reduction in epidermal innervation by CGRP+ fibers in *Ngf*$^{+/-}$ and *Tnfr1*$^{+/-}$;*Ngf*$^{+/-}$ mice compared to *Tnfr1*$^{+/-}$ mice. As predicted, the skin of *Tnfr1*$^{-/-}$;*Ngf*$^{+/-}$ mice exhibited peptidergic fiber innervation comparable to that of *Tnfr1*$^{+/-}$ mice, suggesting that TNFR1 opposes NGF-mediated growth of peptidergic fibers in the periphery.

We performed behavioral analyses on *Tnfr1*$^{+/-}$, *Ngf*$^{+/-}$, *Tnfr1*$^{+/-}$;*Ngf*$^{+/-}$, and *Tnfr1*$^{-/-}$;*Ngf*$^{+/-}$ mice to determine whether modulating nociceptor circuitry translated to changes in pain perception. *Ngf*$^{+/-}$ and *Tnfr1*$^{+/-}$;*Ngf*$^{+/-}$ mice were less sensitive than *Tnfr1*$^{+/-}$ mice when performing the hot plate task at 50°C, 52.5°C, and 55°C (Figure 7P). As expected, the latency to onset of rapid, sustained grooming was rescued in *Tnfr1*$^{-/-}$;*Ngf*$^{+/-}$ mice when compared to the less sensitive *Ngf*$^{+/-}$ or *Tnfr1*$^{+/-}$; *Ngf*$^{+/-}$ mice. These loss-of-function data indicate that ablation of TNFR1 can sensitize TrkA signaling in nociceptors and thereby compensate for reduced NGF availability.

In addition to our genetic approach, we sought to use a biochemical approach to definitively test whether TNFR1 indeed functions as a bona fide antagonist of NGF-TrkA signaling in developing nociceptors. To test this, we cultured E14.5 sensory neurons in NGF for 24 hr and assayed for the activation of signaling molecules downstream of NGF-TrkA. We found that *Tnfr1*$^{-/-}$ sensory neurons exhibit increased activation of the NGF-TrkA downstream effectors extracellular signal-regulated kinase 1/2 (ERK1/2) after 24 hr in vitro relative to WT sensory neurons (Figure 7Q). Moreover, we conducted an NGF deprivation time course to measure whether *Tnfr1*$^{-/-}$ sensory neurons exhibit sustained activation of the NGF-TrkA pathway after the removal of NGF. Indeed, *Tnfr1*$^{-/-}$ neurons maintained hyperactivation of phosphorylated (P)-ERK1/2, at intervals after NGF withdrawal (Figure 7R) despite decaying at the same rate (Figure S6). These data more directly demonstrate that TNFR1 attenuates the strength of NGF-TrkA trophic signals in developing nociceptors.

DISCUSSION

This study describes dual roles for TNF-α and TNFR1 signaling in the coordination of peptidergic and nonpeptidergic nociceptive circuits during peripheral nervous system development through forward and reverse signaling paradigms. We demonstrate that, as early as E14.5 and as late as P30, forward TNF-α-TNFR1 signals are required to negatively regulate the neurite growth, differentiation, and excitability of peptidergic nociceptors. In contrast, TNFR1-TNF-α reverse signaling is required for full target innervation by and excitability of nonpeptidergic nociceptors between P14 and P30.
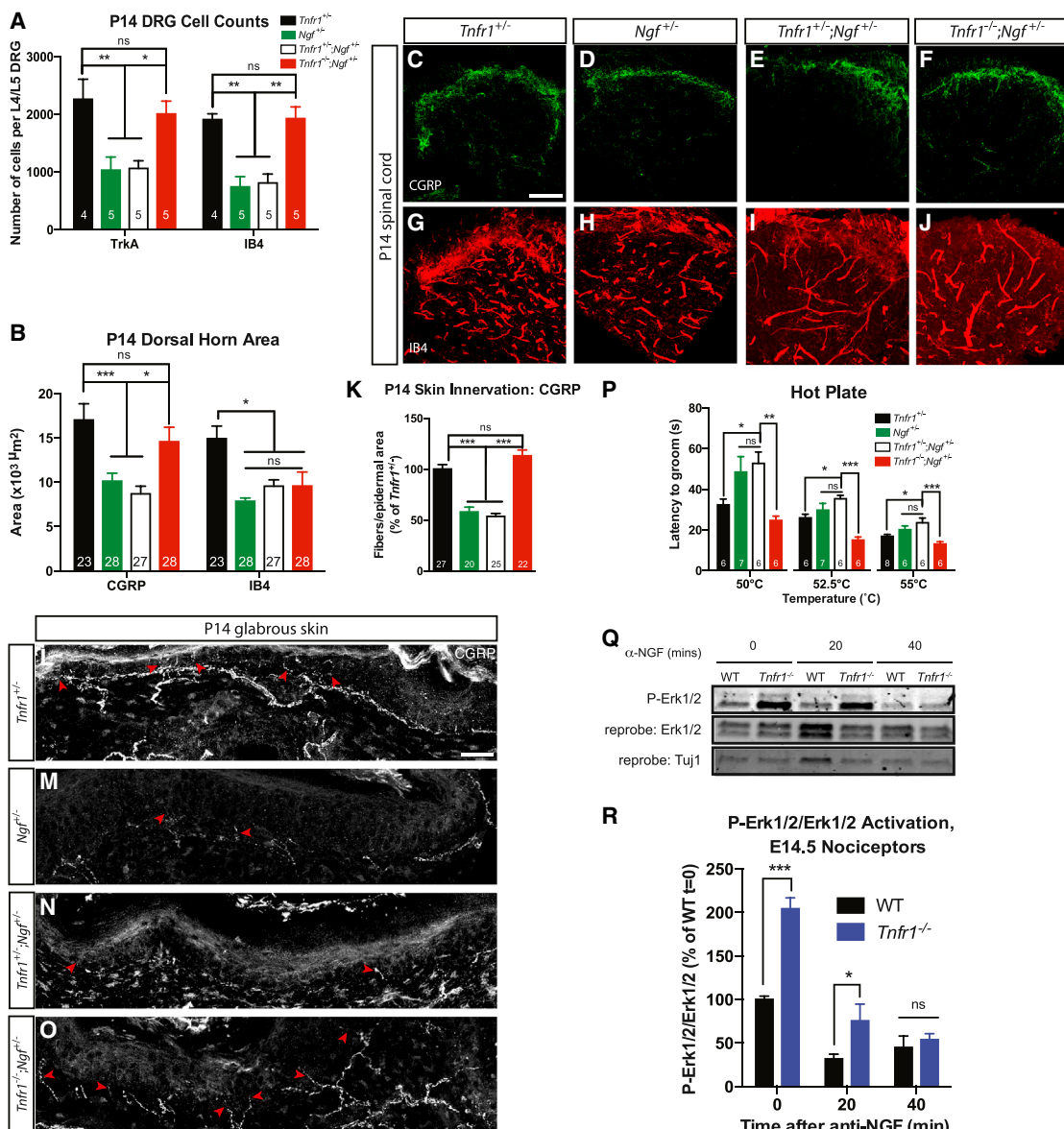
Figure 7. Loss of *Tnfr1* Can Compensate for Heterozygous *Ngf* Deletion

(A) Quantification of the number of TrkA+ and IB4+ neurons per L4/L5 DRG at P14. One DRG used per animal; the number of DRGs is indicated.

(B–J) Analysis of the P14 L4/L5 spinal cord dorsal horn innervation density of CGRP (C–F) and IB4 (G–J) fibers and quantification (B). Five animals were analyzed per genotype; number of sections analyzed is indicated. Scale bar represents 50 μm.

(K–O) CGRP+ fiber density in P14 footpad (thick) glabrous skin. (L–O) Representative images of CGRP+ peptidergic nociceptor fibers invading the hindpaw footpad of P14 mice. Arrowheads point to invading fibers. Scale bar represents 25 μm. (K) Quantification of peptidergic nociceptor skin innervation. Five mice were analyzed per genotype.

(P) Hot plate behavioral analysis at 50°C, 52.5°C, and 55°C. Behavioral analysis performed on mice from a mixed, B6;129 background. Mice analyzed are littermate controls except *Ngf*^{+/−}, which are background matched.

(Q and R) Western blot (Q) and quantification (R) showing the decay in P-ERK1/2 signal after E14.5 nociceptors from WT and *Tnfr1*^{−/−} mice were deprived of NGF via incubation with anti-NGF for the indicated times. Nociceptors were cultured in 45 ng/ml NGF for 1 day in vitro. Data are from three independent experiments using cultured nociceptors from four to six mice per experiment per genotype. Two-way ANOVA, Holm-Šidák posttest.

See also Figure S6. Statistics determined by one-way (K and P) or two-way (A and B) ANOVA, Tukey posttest. Data represent mean ± SEM. ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001.

We propose that these differential functions are principally dependent on the complement of RTK signaling and trophic factor responsiveness resident in different nociceptor popula- tions, specifically NGF-TrkA and NRTN-GFRα2-Ret in pepti- dergic and nonpeptidergic nociceptors, respectively (Figures 8A and 8B).
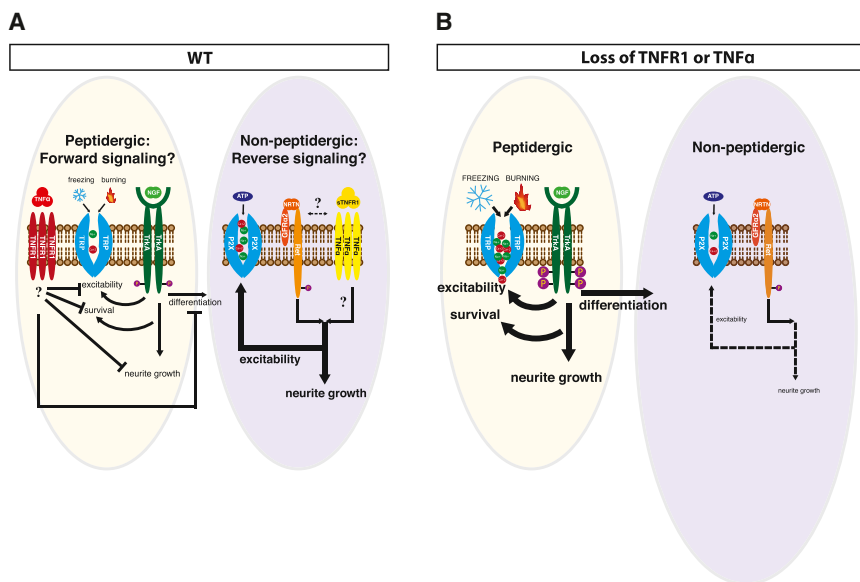
Figure 8. Model of TNF-α-TNFR1 Signaling Properties in NGF-Responsive and NRTN-Responsive Peptidergic and Nonpeptidergic Nociceptors

(A) Model of TNFR1 and TrkA signaling events in WT animals. TNF-α-TNFR1 forward signals suppress NGF-TrkA-dependent excitability, neurite growth, survival, and differentiation. These signals lead to proper specialization of some TrkA+ peptidergic nociceptors as Ret+ nonpeptidergic neurons, which are approximately equal in number to peptidergic cells in young adulthood (P30). Nonpeptidergic nociceptors are dependent on TNFR1-TNF-α reverse signaling for maximal neurite outgrowth and excitability.

(B) Loss of TNF-α or TNFR1 signaling causes a gain of function of NGF-TrkA signals, hyperactivating TrkA excitability, neurite growth, cell survival, and differentiation pathways (thick arrows). Consequently, more TrkA+ neurons are driven to toward a nonpeptidergic fate earlier during development, which results in roughly 50% more IB4+ neurons by young adulthood (P30). In contrast, nonpeptidergic nociceptors are deficient in excitability and NRTN-dependent neurite growth in the absence of TNF-α or TNFR1 (dashed arrows), possibly through loss of a reverse signaling mechanism.

TNF-α-TNFR1 Forward Signaling Attenuates NGF-TrkA-Dependent Constructive Processes in Peptidergic Nociceptors

Our in vivo and in vitro results provide evidence that TNF-α and TNFR1 are required for proper development of nociceptive circuits through antagonism of NGF-TrkA signaling. Several lines of evidence support this assertion, including the following: (1) In vivo, $Tnfr1^{-/-}$ and $Tnf^{-/-}$ mice exhibit an increased density of nociceptive fibers both peripherally and centrally (Figures 2A–2D and 3). (2) In vitro, $Tnfr1^{-/-}$ and $Tnf^{-/-}$ neurons are more sensitive to NGF-mediated neurite growth (Figures 2E–2I). (3) In vitro calcium imaging suggests that TNF-α-TNFR1 signaling normally suppresses the sensitivity of several TRP channels in TrkA+ peptidergic nociceptors because TrkA+ nociceptors are hyperexcitable in the absence of TNF-α-TNFR1 signaling (Figure 5A) but can be rescued via reactivation of this pathway (Figure 5B). (4) We show that homozygous deletion of $Tnfr1$ is sufficient to compensate for neurite growth and behavioral pain deficits caused by the loss of a single Ngf allele (Figure 7). (5) Finally, we provide biochemical evidence of TNFR-RTK antagonism in neurons, by showing that TNFR1 suppresses NGF-TrkA activation (Figures 7Q and 7R).

Our findings that TNF-α-TNFR1 signaling and NGF-TrkA signaling mutually oppose one another in nociceptors are analogous to the broad antagonistic function of p75NTR in sympathetic neurons (Kaplan and Miller, 2000; Deppmann et al., 2008). For instance, p75NTR prevents axon overgrowth (Yeo et al., 1997; Singh et al., 2008), modulates electrical properties (Luther and Birren, 2009), and expedites developmental cell death (Deppmann et al., 2008) in sympathetic neurons. Although p75NTR does not appear to play this role in sensory neurons (Lee et al., 1992), we suggest that TNF-α and TNFR1 may have assumed the antagonistic role toward NGF-TrkA processes in

developing nociceptors. Indeed, loss of $Tnfr1$ leads to hyperactivation of trophic pathways such as P-ERK1/2, consistent with an increase in nonpeptidergic nociceptor number and peptidergic nociceptor target innervation, as suggested previously (Zhong et al., 2007; Newbern et al., 2011). This apparently direct TNF-α-TNFR1 suppression of downstream neurotrophic signals is reminiscent of a similar relationship with the insulin receptor (Steinberg et al., 2006). Thus, it is interesting to speculate that crosstalk between RTK and TNFR family members is a general theme in neuroscience and cell biology. More specifically, whether other TNFR family members also serve as prorefinement mechanisms in sensory neurons is an interesting future line of inquiry. DR6 is one likely candidate given its robust enrichment in the DRG (Figure S1A) and its pronounced role in sensory neuron degeneration (Nikolaev et al., 2009). One additional avenue of future investigation will be to ask whether TNFR family members can cooperate to facilitate prorefinement functions in the somatosensory system.

TNF-α and TNFR1 Enhance Nonpeptidergic Nociceptor Growth and Excitability

Despite increases in the number of IB4+ neurons in mice lacking TNF-α or TNFR1, it appears that rather than Ret antagonism, TNFR1 signaling cooperates with Ret in nonpeptidergic nociceptors. Several lines of evidence support this synergistic relationship, including the following. (1) In vivo, a reduction in IB4+ fibers occupying lamina IIi at P14 and P30, despite the presence of more nonpeptidergic neurons in the DRG at P14 and P30 (Figures 4F–4M). (2) In vitro, DRG explants from $Tnfr1^{-/-}$ or $Tnf^{-/-}$ mice display impaired neurite outgrowth in response to NRTN (Figures 4N–4P and 4S). (3) Addition of sTNFR1, but not soluble tumor necrosis factor (sTNF), rescues the impaired neurite outgrowth of explants cultured in NRTN (Figures 4Q–4S). (4)

In vitro calcium imaging assays reveal that $Tnf^{-/-}$ and $Tnfr1^{-/-}$ nonpeptidergic nociceptors are hypoexcitable to acute ATP and capsaicin stimulation (Figures 5D and 5E). (5) The inability of a $Tnfr1$ deletion to rescue deficient nonpeptidergic central projections to lamina IIi caused by Ngf heterozygosity (Figures 7B and 7G–7J). These results point to a dichotomous function for TNF-α and TNFR1 signals in two populations of functionally distinct neurons and suggest that TNF-α and TNFR1 may enhance growth and excitability through reverse signaling.

What is the molecular basis for predominantly TNF-α-TNFR1 forward signaling in peptidergic nociceptors but TNFR1-TNF-α reverse signaling in nonpeptidergic nociceptors? One possibility is that contextual molecular differences bias production of sTNF-α or sTNFR1. For instance, just as NGF-TrkA signals upregulate the expression of a disintegrin and metalloprotease 17 (ADAM17) in sympathetic neurons (Kommaddi et al., 2011), it is possible that a similar paradigm exists in TrkA+ sensory neurons. One could envision that strongly trophic TrkA+ nociceptors upregulate ADAM17, which would cleave membrane-bound TNF-α to produce sTNF-α and induce TNF-α-TNFR1 forward signals in peptidergic nociceptors. Upon committing to a nonpeptidergic fate however, ADAM17 or other metalloproteases might preferentially shed TNFR1 from the cell surface to generate sTNFR1. Although further studies on these possibilities are necessary, it is a compelling idea to consider the number of permutations of TNFR family members antagonizing or synergizing with different RTKs to promote vastly different functional outcomes such as what we observe in peptidergic (TrkA+) and nonpeptidergic (Ret+) nociceptors. As such, our findings likely extend beyond neuronal populations specifically, and to RTK and TNFR family member function, generally.

Cell Fate Specification of TrkA+ Sensory Neurons Is Coordinated by TNF-α-TNFR1 Signaling

All newly born nociceptors are TrkA+ but mature to become several diverse subpopulations expressing various combinations of the RTKs TrkA, Ret, and Met during adulthood (Lallemend and Ernfors, 2012; Liu and Ma, 2011; Gascon et al., 2010; Luo et al., 2007). These fate specification events are reported to be dependent on hierarchal NGF-TrkA modulation of the RTK, Ret, and the transcription factor Runx1 (Lallemend and Ernfors, 2012; Gascon et al., 2010; Luo et al., 2007; Chen et al., 2006b). Thus, it stands to reason that perturbing TrkA signals in developing sensory neurons would perturb the fate specification of several different nociceptor lineages. How then is such remarkable diversity coordinated from the standpoint of an early TrkA-expressing nociceptor? We propose that TNFR1 plays a pivotal role in the specification of different nociceptor subtypes based on the following observations: (1) Elimination of TNF-α-TNFR1 signaling leads to higher numbers of TrkA+ nociceptors in adulthood, consistent with previous reports that TNF-α serves a death-promoting developmental role in sensory neurons (Figure 4A) (Barker et al., 2001). (2) Loss of TNFR1 or TNF-α also results in a higher proportion of nociceptors moving toward a nonpeptidergic fate commitment at P0 (Figures 4B–4D). (3) Consistent with this idea, at P14 and P30 there are more IB4+ neurons in $Tnfr1$ and Tnf knockout animals than WT mice in the L4/L5 DRG (Figure 4E).

Previous work has demonstrated that loss of key transcription factors impairs differentiation and function of sensory neurons (Chen et al., 2006a, 2006b; Maricich et al., 2009; Wende et al., 2012). Although the importance of TrkA and Met in regulating nociceptor differentiation has already been established (Luo et al., 2007; Gascon et al., 2010), to our knowledge TNFR1 is the only non-RTK single-pass transmembrane protein reported to influence sensory neuron fate specification. We find that loss of TNF-α-TNFR1 signaling is more akin to enhancement of TrkA-dependent differentiation as more nonpeptidergic nociceptors are generated in the absence of TNF-α or TNFR1. These observations are consistent with our biochemical data that reveal loss of TNFR1 leads to hyperactive P-ERK1/2 signaling (Figures 7Q and 7R) and previous reports implicating ERK/mitogen-activated protein kinase signals in fate specification (Zhong et al., 2007). Importantly, although we specifically focus on the development of nonpeptidergic nociceptors derived from TrkA+ precursors, there are several other distinct populations of cells derived from TrkA+ precursors that are likely affected by loss of TNF-α or TNFR1 such as those expressing mrg family members regulating pain, itch, or even low threshold mechanosensation (Dong et al., 2001; Liu et al., 2009; Li et al., 2011; Vrontou et al., 2013; Abraira and Ginty, 2013). In future studies it will be interesting to examine how TNF-α and TNFR1 influence the development and specification of several important sensory neuron subtypes critical for varied modes of tactile perception.

TNF-α-TNFR1 Forward Signaling Gates NGF-TrkA Signaling to Suppress Pain

Our data suggest that TNFR1 acts as a gatekeeper on nociceptive neurons where it exerts control over several processes underlying stimulus perception. Importantly, these data also imply that TNF-α-TNFR1 signaling within nociceptors is, in turn, controlled by the levels of TNF-α produced by tissues that nociceptors innervate such as the skin and spinal cord. TNF-α-TNFR1 signals modify nociceptor information processing by attenuating NGF-TrkA signals and refining components of the primary nociceptive circuit such that nociceptors properly interpret painful touch. This study and findings from other groups have demonstrated that elimination of NGF or TrkA signaling results in nociceptive impairment (Crowley et al., 1994; Silos-Santiago et al., 1995; Indo et al., 1996). Conversely, excessive NGF-TrkA activation causes nociceptor sensitization (Pezet and McMahon, 2006; Lane et al., 2010). Thus, deletion of Tnf or $Tnfr1$ is analogous to increasing the sensitivity of nociceptors to NGF stimulation, effectively increasing the "gain in pain" signals that are transmitted to the CNS (Figure 6), as suggested previously (Woolf and Salter, 2000). In support of this notion, we find that loss of TNFR1 can rescue the defective nociceptive perception observed in mice heterozygous for Ngf. This rescue can be observed for cell number, central projection, and peripheral innervation density as well as behavior (Figure 7). Importantly, we present biochemical evidence that suggests these phenotypes are due to hyperactive NGF-TrkA signals in nociceptors.

This study supports the emergent concept that development of the peripheral nervous system requires a balance of

interacting prorefinement and proconstructive cues. When this equation becomes unbalanced, we observe disorders such as congenital insensitivity to pain or TNFR-associated periodic syndrome, an autoinflammatory disease marked by recurrent pain and fever (Ozen and Bilginer, 2014). Although the idea that TNFR family members negatively regulate the Trk family of RTKs is not a new idea, the argument that the infamously proinflammatory TNF-α-TNFR1 pathway opposes pain perception is significant. In fact, we report that nearly every major hallmark of the NGF-TrkA pathway is augmented when *Tnfr1* or *Tnf* is ablated, suggesting a broad, general, and robust antagonism of TrkA signaling in nociceptors. Surprisingly, although TNF-α and TNFR1 are analgesic signals in TrkA+ nociceptors, they serve an algesic role in Ret+ nociceptors, highlighting the importance of molecular context in the formation of developing neural circuits.

EXPERIMENTAL PROCEDURES

Animals

All experiments were carried out in compliance with the Association for Assessment of Laboratory Animal Care policies and approved by the University of Virginia Animal Care and Use Committee.

WT mice are on a B6;129 mixed background. *Tnfr1a*$^{-/-}$ and *Tnf*$^{-/-}$ mice were purchased from The Jackson Laboratory and backcrossed to a B6;129 mixed background for four or more generations. *Ngf*$^{+/-}$ mice were a gift from David Ginty and were maintained on a B6;129s mixed background.

In Vitro Calcium Imaging

Calcium imaging was performed with Fluo-4 AM (Life Technologies) as described previously (Suo et al., 2014), with some modifications. Briefly, mass cultures of nociceptors were established in NGF, loaded with Fluo-4 AM, and then imaged before acute treatment with 1 μM capsaicin (MP Biomedicals), 100 μM L-menthol (MP Biomedicals), or 100 μM mustard oil (Acros Organics). The change in fluorescence after treatment with a compound was measured relative to baseline fluorescence, and a relative fold change was computed. One chemical treatment was used per experiment. P14 cultures were also loaded with IB4-568 (Life Technologies).

Behavioral Assays

Mice were tested between 4 and 8 weeks (Figure 6), between 4 and 6 weeks (Figure 7P), or between 6 and 7 weeks (Figure S5) during the light cycle. Before testing, mice were habituated to handling. Behavioral experiments in Figure 6 were performed on mixed background matched (B6;129s), nonlittermate controls. In Figure 7 behavioral experiments, *Ngf*$^{+/-}$ rescue experiments were performed with littermate controls except for *Ngf*$^{+/-}$ mice, which were background matched on the B6;129 mixed background.

Behavioral tests were performed with at least 20 min between tests, and no more than three tests per day. One trial per mouse is reported for each data point.

Tail Flick

Mice were manually restrained while the posterior 1/3 of the mouse's tail was submerged in a water bath (Fisher Scientific Isotemp) maintained at the designated temperature ±0.3°C until mouse flicked or reacted to the temperature.

Hot Plate

Mice were placed on plates maintained at the given temperature (Columbus Instruments Hotplate analgesia meter) and restricted to move within the area of an inverted 1,000 ml beaker. The latency to vigorously lick/groom was recorded.

Hargreaves

A mouse was manually restrained, and its hindpaw was placed over an infrared light source (Ugo Basile) until reflexive removal of paw.

von Frey

Mice were placed on a thin mesh screen and restrained within slightly opaque red containers during the test duration. Mechanical filaments of varying diameter (Bioseb Touch Test) were administered to hindpaws beneath the apparatus, and the percent response of paw lifting per five trials per filament was recorded.

Acetone Drop

We applied 50 μl of acetone to the hindpaw and permitted it to evaporate for 10 s. The duration of grooming per 120 s after the evaporation was recorded. Grooming was judged as forepaw/hindpaw/stomach licking, facial grooming, and hindpaw dragging.

Quantitation of Images

Quantification of skin innervation was performed as described previously (Zylka et al., 2005). DRG analyses were performed largely as described previously (Luo et al., 2007). Spinal cord quantification analyses were performed as described previously (Guo et al., 2011).

RT-PCR, Immunostaining, Cell Culture, Explants, Dissociated Neuron Neurite Growth, and Western Blotting

Detailed protocols are in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2014.04.009.

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