NEUROPILIN-1 IS A CO-RECEPTOR FOR NGF AND TRKA-EVOKED PAIN

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ABSTRACT

Nerve growth factor (NGF) monoclonal antibodies are one of the few patient-validated non-opioid treatments for chronic pain, despite failing to gain FDA approval. Herein, we demonstrate that neuropilin-1 (NRP1) is a co-receptor for NGF that potentiates tropomyosin-related kinase A (TrkA) signaling. NGF binds NRP1 with nanomolar affinity. NRP1 and its associated adaptor protein GIPC1 are coexpressed with TrkA in nociceptors, particularly in human tissues. NRP1 inhibitors prevent NGF-stimulated action potential firing of human and mouse nociceptors and abrogate NGF-evoked and inflammatory nociception in mice. NRP1 knockdown blunts NGF-stimulated TrkA phosphorylation, kinase signaling and transcription, whereas NRP1 overexpression enhances signaling. As well as interacting with NGF, NRP1 forms a heteromeric complex that chaperones TrkA from the biosynthetic pathway to the plasma membrane and signaling endosomes. NRP1 thereby enhances NGF-induced TrkA dimerization, endocytosis and signaling. Knockdown of GIPC1, a PDZ-binding protein that scaffolds NRP1 and TrkA cargo to myosin VI, abrogates NGF-evoked nociception. We identify NRP1 as a previously unrecognized co-receptor necessary for NGF/TrkA pain signaling by direct NGF binding and chaperoning TrkA to the plasma membrane and signaling endosomes *via* the adaptor protein GIPC1 - a discovery that offers a long-awaited alternative to block NGF-evoked pain.

INTRODUCTION

The first discovered growth factor, nerve growth factor (NGF), was identified by its ability to stimulate growth of sympathetic neurons[1]. Tropomyosin-related kinase A (TrkA), a receptor tyrosine kinase (RTK), mediates the neurotrophic actions of NGF[2]. NGF/TrkA signalosomes are transported in a retrograde direction from peripheral nerve terminals to the soma, where they regulate transcription and neuronal development[3,4]. NGF and TrkA are also implicated in pain[5]. $p75^{NTR}$ is a co-receptor for NGF and the immature pro-peptide[6,7] that activates opposing pro-apoptotic signaling pathways[8]. Although NGF and its receptors have been intensively studied in the context of neuronal development and pain, mechanistic gaps in understanding have hampered the successful approval of NGF-directed therapeutics.

Chronic pain afflicts twenty percent of the global population at some point in life, yet is inadequately treated by opioids and non-steroidal anti-inflammatory drugs, which lack efficacy and have life-threatening side effects. NGF/TrkA are one of the few non-opioid targets for chronic pain validated in patients. NGF, which is produced by injured and diseased tissues, activates TrkA on peripheral nociceptors to evoke hyperexcitability and the expression of neuropeptides and ion channels that mediate pain[5]. The central role TrkA and NGF in pain is evident in patients with Hereditary Sensory and Autonomic Neuropathy (HSAN) Type IV and V, where pathological insensitivity to pain results from loss-of-function mutations in TrkA[9] and NGF[10], respectively. Although monoclonal antibodies (mAbs) are analgesic in osteoarthritic patients[11], they lack FDA approval due to worsening joint damage in some individuals[12]. The identification of nociceptor-enriched mediators of NGF-induced pain may facilitate development of non-opioid analgesics, avoiding the adverse effects of systemic NGF sequestration with mAbs.

Transcriptome analyses have identified proteins that are conserved between rodent and human nociceptors, including neuropilin-1 (NRP1)[13]. NRP1 is a type I transmembrane protein discovered for its role in axon guidance[14]. Lacking a catalytic domain, NRP1 does not transduce signals *per se* but rather acts as a co-receptor for unrelated families of proteins, including vascular endothelial growth factor (VEGF-A)[15]. NRP1 is a co-receptor with VEGF receptor 2 (VEGFR2) that enhances VEGF-A-induced blood vessel development. NRP1 overexpression in cancers spurred the development of a mAb, vesencumab/MNRP1685A, to inhibit tumorigenesis[16]. NRP1 is also implicated in VEGF-A-mediated pain[17,18]. Since NGF, a major pain mediator, possesses a prospective NRP1 binding motif, we hypothesized that NRP1 is a co-receptor for NGF and TrkA in nociception.

RESULTS

NRP1 binds human β-NGF

NRP1 is a co-receptor for unrelated families of proteins including neuronal guidance molecules (semaphorins)[19], growth factors (VEGF-A)[15], cell-penetrating peptides[20], and viruses (SARS-CoV-2)[14]. NRP1 ligands share a C-terminal basic motif (C-end rule, “CendR” motif, R/KXXR/K), which interacts with extracellular “b” domains of NRP1[15,19]. Inspection of the amino acid sequence of mature NGF identified prospective ‘CendR’ R/KxxR/K motifs within the C-terminus that are conserved between rodents and humans and known to mediate the interaction of other growth factors with NRP1[20] (**Fig. 1A**). Molecular modeling of βNGF and human NRP1 identified a potential interaction between the CendR motif at the C-terminus of NGF and the b1b2 domain of NRP1. To examine the potential for productive interactions between NGF and NRP1, we used restrained protein-protein docking of a human NGF structure terminating in Arg118 (PDB 4GZ9[21]; **Fig. 1B**) to the AlphaFold-predicted structure of soluble human a1a2b1b2 domains of NRP1. Our modeling supports the hypothesis that these proteins could interact *via* the canonical CendR binding mechanism, similar to the interactions observed for structures of $VEGF_{165}$a CendR bound to the b1 domain[22].

To directly investigate whether NGF binds purified NRP1, microscale thermophoresis (MST) was used to analyze interactions between human NGF and His-tagged human NRP1. NGF interacted with extracellular NRP1 a1a2b1b2 domains with nanomolar affinity (K_d = 65.64 ± 6.66 nM, n=4, **Fig. 1C**). This is comparable to published affinities between purified $VEGF_{165}$a and NRP1 (K_d 9 nM[23] to 120 nM[24]).

NGF binding to NRP1 in living cells at 37ºC was analyzed using a modified bioluminescence resonance energy transfer (BRET)-based assay (**Fig. 1D**). $VEGF_{165}$a (positive control growth factor known to interact with NRP1) or NGF was genetically fused to an 11 amino acid fragment (HiBiT) of nanoluciferase

(NanoLuc). HiBiT has a high affinity for the complementary LgBiT fragment. HiBiT-tagged growth factors were expressed in HEK293T cells, secreted into supernatant, and conjugated to recombinant LgBiT to form full length and catalytically active NanoLuc. Upon addition of furimazine substrate, complemented NanoLuc emits bioluminescence. This supernatant containing HiBiT-tagged growth factor was incubated with cells expressing SnapTag-NRP1 labeled with SNAP-Surface® Alexa Fluor® 488 fluorophore (SnapTag-AF488, +) or lacking fluorophore (-). If proteins are in close proximity (<10 nm), luminescent growth factor can act as a BRET donor to NRP1. As a positive control, BRET was detected between luminescent $VEGF_{165}$a and SnapTag-NRP1 (**Fig. 1E**). Pre-incubation of cells with an excess of $VEGF_{165}$a (10 nM) abolished the BRET signal. No BRET signal was detected when luminescent $VEGF_{165}$a was incubated with cells expressing a binding-dead NRP1 mutant in the b1 domain[25,26]. Importantly, BRET was detected between NGF-HiBiT and SnapTag-NRP1, and pre-incubation with unlabeled $VEGF_{165}$a inhibited this response.

The results from molecular modeling, analysis of interactions between recombinant proteins, and ligand binding studies in intact cells provide evidence that NGF directly binds NRP1 with nanomolar affinity.

Ntrk1/TrkA and _Nrp1_/NRP1 are co-expressed in mouse and human DRG neurons

To determine whether NRP1 could function as an NGF/TrkA co-receptor in nociceptors, TrkA and NRP1 were localized in mouse and human dorsal root ganglia (DRG) by immunofluorescence (protein) and RNAScope® *in situ* hybridization (mRNA). In mice, immunoreactive TrkA was detected in vesicles and immunoreactive NRP1 was localized at the plasma membrane of the same DRG neurons (**Fig. 2A**). *Ntrk1* (TrkA) and *Nrp1* (NRP1) mRNAs colocalized in both mouse and human DRG neurons, which were identified by NeuN immunostaining (**Fig. 2B,C**). In mice, *Ntrk1* mRNA was expressed in 27% and *Nrp1* mRNA was expressed in 30% of the neurons; *Nrp1* was co-expressed in approximately one third of *Ntrk1*-positive neurons (**Fig. 2D**). In humans, *Nrp1* was co-expressed in approximately 93% of *Ntrk1*-positive neurons (**Fig. 2E**). Thus, TrkA and NRP1 are co-expressed in the same neurons in DRG in mouse and human, where NRP1 is appropriately located to control NGF/TrkA signaling.

NRP1 antagonism abrogates NGF-induced ionic currents in mouse and human DRG neurons

NGF/TrkA signaling promotes pain by kinase-mediated phosphorylation and sensitization of ion channels, leading to enhanced sensitivity to mechanical, thermal and chemical stimuli, thus activating voltage-gated ion channels in DRG neurons[5]. Patch-clamp recordings under current-clamp mode were made to determine whether NRP1 is necessary for NGF-induced excitability and ion channel activity in dissociated human DRG neurons. Human NGF (50 nM) increased the number of action potentials elicited by a depolarizing ramp pulse from 0 to 250 pA in 1 s (**Fig. 3A,B**). Pre-incubation with the NRP1 inhibitor EG00229 (30 μM)[25] prevented NGF-induced hyperexcitability (**Fig. 3A,B**). NGF or EG00229 did not affect the resting membrane potential (**Fig. 3C**) or the rheobase, the minimum current necessary to elicit an action potential (**Fig. 3D**). Murine NGF similarly stimulated action potential firing in mouse DRG nociceptors (Fig. 3E-G). Pre-incubation with EG00229 blocked NGF-induced excitability (**Fig. 3E,F**), while NGF has no effect on resting membrane potential or rheobase (**Fig. 3G,H**).

Since both Ca^{2+} and Na^{+} channels have critical roles in controlling the excitability of sensory neurons, we further tested whether NGF increased the activity of voltage-gated ion channels by making patch-clamp recordings under the voltage-clamp mode in dissociated mouse DRG. NGF (50 nM) increased total Ca^{2+} currents (**Fig. 3I**) and current density (**Fig. 3J,K**) by ~50% when compared to vehicle (DMSO)-treated cells. EG00229 prevented NGF-induced activation of Ca^{2+} currents, but had no effect on Ca^{2+} currents in unstimulated DRGs (**Fig. 3J,K**). Half-maximal activation and inactivation ($V_{1/2}$) potential, as well as slope factor values (k) for activation and inactivation, were not different between the conditions tested, except for an ~8-mV hyperpolarizing shift in Ca^{2+} channel activation induced by EG00229 when compared to DMSO- and NGF-treated DRGs (**Fig. 3L, Supplementary Table 1**). Similarly, EG00229 normalized NGF-induced increases in Na^{+} currents (**Fig. 3M**). NGF caused a two-fold increase in Na^{+} current density (**Fig. 3N**) and peak current density (**Fig. 3O**) when compared to vehicle (DMSO)-treated DRG neurons. EG00229 abolished these effects of NGF but had no effects on Na^{+} currents in unstimulated DRGs (**Fig. 3N,O**). There were no detectable changes in the voltage-dependence of activation and inactivation between the conditions tested, except for a ~10-mV depolarizing shift in the $V_{1/2}$ of inactivation of EG00229 and NGF-treated cells compared to control (**Fig. 3P**). These data implicate voltage-gated Ca^{2+} and Na^{+} channels as

downstream effectors of NRP1-mediated NGF signaling. Thus, NRP1 is necessary for NGF-induced sensitization of nociceptors in rodents to humans.

NRP1 antagonism inhibits NGF-induced nociception in mice

NGF injection is known to induce pain in humans and rodents[5]. In addition to sensitizing ion channels, NGF/TrkA signaling also promotes chronic pain through the upregulation of genes that mediate pain (*e.g.*, substance P, transient receptor potential vanilloid 1)[5]. To test whether NRP1 is necessary for NGF-induced nociception, murine NGF (50 ng) was administered to mice by intraplantar (i.pl., 10 µl) injection (**Fig. 4A**). A human mAb against the b1b2 domain of NRP1 (vesencumab[16], 7 µg/10 µl) or control IgG (7 µg/10 µl), or small molecule NRP1 inhibitor EG00229 (30 µM/10 µl)[25] or compound 5 (Cpd-5; 30 µM/10 µl)[27] or vehicle (PBS, 10 µl) was co-administered (i.pl.) with NGF. Withdrawal responses of NGF-injected (ipsilateral) hindpaws to stimulation with von Frey filaments (VFF) and radiant heat were assessed to evaluate mechanical allodynia and thermal hyperalgesia, respectively. In control experiments, NGF decreased the withdrawal threshold to VFF stimulation and the latency of withdrawal to thermal stimulation within 30 min for at least 4 h, indicating mechanical allodynia and thermal hyperalgesia (**Fig. 4B-G**). Vesencumab and EG00229 strongly inhibited NGF-induced mechanical allodynia for 2 h and Cpd-5 inhibited mechanical allodynia for 1 h (**Fig. 4B-C**). Vesencumab, EG00229 and Cpd-5 also inhibited NGF-induced thermal hyperalgesia for 1-2 h (**Fig. 4E-G).** None of the inhibitors affected the withdrawal responses of the ipsilateral paw to mechanical or thermal stimuli in mice that did not receive NGF (**Fig. 4B-G**).

NGF mediates inflammatory pain[5], providing an opportunity to determine whether NRP1 is necessary for the pronociceptive actions of endogenous NGF. Complete Freund's Adjuvant (CFA, 1 mg/ml, 10 µl, i.pl.) was injected into the hindpaw. Vesencumab mAb (7 µg/10 µl) or IgG Ctrl (7 µg/10 µl), or EG00229 (30 µM/10 µl) or PBS (10 µl) (i.pl.) was injected into the inflamed paw 48 h after CFA (**Fig. 4H**). CFA induced sustained mechanical allodynia and thermal hyperalgesia (**Fig. 4I-L**). Vesencumab, but not EG00229, reduced thermal hyperalgesia 1 h after treatment (**Fig. 4K,L**). Vesencumab inhibited mechanical allodynia compared to IgG control, with a maximal effect at 1 h, lasting up to 2 h post-injection (**Fig. 4I**). EG00229 also partially reversed mechanical allodynia compared with vehicle, with a maximum inhibitory effect at 1 h, lasting for up to 4 h (**Fig. 4J**). Thus, NRP1 is necessary for the pronociceptive actions of exogenous and endogenous NGF in mice.

NRP1 controls NGF and TrkA kinase signaling

NGF primarily signals through TrkA[28], a 'crab claw'-like RTK where NGF binds to extracellular immunoglobulin-like domains to induce conformational changes throughout the RTK dimer that initiate auto- and trans-phosphorylation of intracellular tyrosine residues (*e.g.,* Y490, Y785). To determine whether NRP1 controls NGF-stimulated phosphorylation of TrkA, a proximal and necessary component of TrkA signaling, dissociated mouse DRG neurons were stained with a phospho-specific antibody to TrkA phosphorylation at residue Y785 (equivalent to Y794 in mouse TrkA). NGF (100 nM, 15 min) stimulated a ~1.4-fold increase in the intensity TrkA phospho-Y785 immunofluorescence compared to vehicle (**Fig. 5A,B**). Pre-incubation with EG00229 (30 µM, 30 min) prevented NGF-stimulated phosphorylation of TrkA but had no effect on the basal unstimulated phosphorylation. These results support the hypothesis that NRP1 is necessary for NGF-induced activation of TrkA.

Activated TrkA stimulates extracellular signal-regulated kinase (ERK) phosphorylation, as well as AKT and phospholipase Cγ. In addition to triggering neuronal development, TrkA-induced ERK signaling contributes to NGF-induced sensitization of nociceptors and pain[5]. The contribution of NRP1 to ERK activation was monitored in CAD.a cells, a neuron-like cell line modified from the Cath.a catecholaminergic cell line obtained from a murine tumor[29]. While CAD.a cells lack TrkA expression, they contain $p75^{NTR}$ and NRP1 (**Supplementary Fig. 1**). To probe NGF/TrkA ERK signaling with high spatial and temporal resolution, Förster resonance energy transfer (FRET) EKAR biosensors targeted to the cytosol or nucleus were coexpressed with human TrkA in CAD.a cells. EKAR biosensors contain a reversible substrate sequence separated by two fluorophores (**Fig. 5C**). NGF activated ERK in the cytosol and nucleus within 5 min for at least 20 min (**Fig. 5D**). NGF-induced ERK activation was concentration-dependent, with a higher potency for nuclear than cytosolic ERK (**Fig. 5E,F, Supplementary Table 2**). Pre-incubation with EG00229 (30 µM) did not affect NGF activation of cytosolic ERK (**Fig. 5E**), but significantly reduced the potency for

NGF activation of nuclear ERK (**Fig. 5F, Supplementary Table 2**; NGF EC_{50}: DMSO control, ~8 pM; EG00229 ~98 pM; paired t-test, *P*=0.023).

The contribution of NRP1 to NGF-induced ERK signaling was also investigated by NRP1 overexpression with TrkA in HEK293T cells, which express low levels of these proteins. As in CAD.a cells, NGF activated ERK in the cytosol and nucleus of HEK293T cells (**Fig. 4G**). While NRP1 co-expression did not influence cytosolic ERK signaling (**Fig. 5H**), NRP1 expression significantly enhanced activation of nuclear ERK in response to low (1 pM) NGF concentrations (**Fig. 4I**). The outcomes of nuclear ERK signaling were studied by expression of a transcriptional luciferase reporter, where a luminescent protein is produced downstream of an ERK promoter (**Fig. 4C**). NGF stimulated concentration-dependent ERK transcriptional activity with significantly higher potency in cells overexpressing TrkA and NRP1 compared to cells expressing TrkA alone (**Fig. 5J, Supplementary Table 2**; NGF EC_{50}: TrkA alone, 214 pM; TrkA + NRP1, 71 pM; paired t-test, *P*=0.0004). NGF did not stimulate ERK transcriptional activity in cells expressing NRP1 alone, confirming lack of inherent signaling capability of NRP1 (**Fig. 5J**). These results support the notion that NRP1 enhances NGF-induced TrkA activation and signaling by pathways that underpin nociception. We next sought to determine the mechanism of NRP1-mediated potentiation of NGF/TrkA signaling.

NRP1 is a chaperone that forms a complex with TrkA to control trafficking from the biosynthetic pathway to the plasma membrane

In addition to directly binding NGF, NRP1 could amplify NGF/TrkA signaling by receptor/co-receptor interactions with TrkA, akin to its co-receptor function with VEGFR2[30]. Cell surface expression of human TrkA and NRP1 was measured by specific substrate-based labeling of enzymatic tags genetically fused to the extracellular N-terminus of either receptor. SnapTag-TrkA and HaloTag-NRP1 were expressed in HEK293T and CAD.a cells and covalently labeled with membrane-impermeant AlexaFluor® substrates, thereby selectively labeling receptors transported to the cell surface. SnapTag-TrkA and HaloTag-NRP1 were highly co-localized at the cell surface of HEK293T and CAD.a cells, with the latter also showing a high level of co-localization in subcellular compartments (**Fig. 6A**). Labeling specificity was confirmed in cells expressing TrkA or NRP1 alone (**Supplementary Fig. 2**).

To investigate the formation of a heteromeric complex between TrkA and NRP1, BRET was measured in HEK293T cells expressing NanoLuc-NRP1 and SnapTag-TrkA. Extracellular N-terminal NanoLuc acts as an energy donor to excite a nearby SnapTag. Measurement of BRET between NanoLuc-NRP1 and increasing levels of SnapTag-TrkA revealed a hyperbolic relationship, indicative of assembly of a heteromeric complex between NanoLuc-NRP1 and SnapTag-TrkA (**Fig. 6B,C**). As a positive control, a hyperbolic BRET signal relationship was also detected between NanoLuc-$p75^{NTR}$, which is known to interact with TrkA[5], and increasing levels of SnapTag-TrkA. In contrast, there was a linear BRET signal between NanoLuc-NRP1 and increasing levels of SnapTag-calcitonin-like receptor (CALCRL), an unrelated transmembrane receptor. To verify whether this relationship was observed with protein expression, data from a representative experiment quantifying cell surface SnapTag labeling also demonstrated a hyperbolic curve between TrkA and NRP1 (**Fig. 6C**). These results suggest that NRP1 and TrkA co-localize at the plasma membrane as a heteromeric complex.

Recruiting TrkA to the plasma membrane would enable increased access to extracellular growth factor. The effect of NRP1 on the cell surface expression of TrkA was determined using the membrane-impermeant SnapTag fluorophore to selectively label cell surface TrkA (**Fig. 6D**). NRP1 co-expression increased level of TrkA at the surface in HEK293T cells by 150 ± 43% and of CAD.a cells by 109 ± 4% (**Fig. 6E-G**). Enhanced bystander BRET, which capitalizes on the endogenous affinity of pairs of *Renilla*-tagged proteins to boost sensitivity[31], was developed to quantify the effects of NRP1 expression on the localization of TrkA in different subcellular compartments of living cells. TrkA tagged on the C-terminus with Renilla luciferase (Rluc, BRET) was coexpressed in HEK293T or CAD.a cells with proteins resident of the plasma membrane (CAAX), early endosomes (Rab5a), recycling endosomes (Rab4a) and the cis-Golgi apparatus (Giantin) tagged with *Renilla* green fluorescent protein (RGFP) (**Fig. 6H**). In HEK293T cells, NRP1 expression significantly increased BRET between TrkA-Rluc8 and RGFP-CAAX and significantly decreased BRET between TrkA-Rluc8 and tdRGFP-Rab5a, tdRGFP-Rab4a and tdRGFP-Giantin (**Fig. 6I**). In CAD.a cells, NRP1 expression did not affect BRET between TrkA-Rluc8 and RGFP-CAAX but significantly decreased BRET between TrkA-Rluc8 and tdRGFP-Rab5a, tdRGFP-Rab4a and tdRGFP-

Giantin (**Fig. 6J**). These results indicate that NRP1 expression causes a redistribution of TrkA from subcellular regions involved in receptor recycling or *de novo* export to the plasma membrane, consistent with a chaperone function. Control studies confirmed NRP1 was successfully expressed using a HaloTag label, where NRP1 co-expression had no significant effect on overall TrkA expression (**Supplementary Fig. 3A-D**). These results suggest that NRP1 acts as a chaperone for TrkA.

NRP1 enhances NGF-induced TrkA trafficking and dimerization

Upon NGF stimulation, TrkA traffics to endosomes by clathrin-dependent and -independent mechanisms, a process known to further enable TrkA signaling through NGF/TrkA signalosomes that are retrogradely transported in endosomes or multivesicular bodies to the soma of sympathetic neurons mediate the neurotrophic actions of NGF[3,4]. To determine the effects of NRP1 on NGF-evoked endocytosis of TrkA, BRET was measured between TrkA-Rluc8 and plasma membrane marker RGFP-CAAX (**Fig. 7A**). NGF caused a concentration-dependent decrease in TrkA-Rluc8 and RGFP-CAAX BRET (EC_{50} ~870 pM), with 10-100 nM NGF inducing maximal decrease within 5 min that was sustained for 20 min, consistent with TrkA endocytosis (**Fig. 7B, Table 2**). Hypertonic (0.45 M) sucrose or the clathrin inhibitor pitstop 2 (30 μM) prevented NGF-induced endocytosis of TrkA (**Fig. 7C**). Co-expression of NRP1 with TrkA enhanced NGF-stimulated endocytosis of TrkA (**Fig. 7D**). This effect of NRP1 was observed at higher NGF concentrations (>10 nM), with minimal effect on the potency of NGF-induced endocytosis of TrkA (**Table 2**). NGF also stimulated concentration-dependent removal of TrkA from the plasma membrane of CAD.a cells (EC_{50} ~490 nM; pEC_{50} = 9.31 ± 0.18, n=4) that was inhibited by hypertonic sucrose and pitstop 2 (**Fig. 7E-G**). Knockdown of endogenous NRP1 with siRNA significantly inhibited NGF-induced endocytosis of TrkA in CAD.a cells (**Fig. 7G**). NRP1 knockdown was confirmed at a protein level using immunofluorescence in CAD.a cells, while there was no effect on TrkA expression (**Supplementary Fig. 3E-F**). Thus, whereas NRP1 overexpression enhances agonist-stimulated endocytosis of TrkA in HEK293T cells, NRP1 knockdown has the opposite effect in CAD.a cells. The results are consistent with a chaperone role for NRP1 in NGF-stimulated endocytosis of TrkA.

Dimerization of TrkA is pivotal for NGF/TrkA signaling[5]. The contribution of NRP1 to TrkA oligomerization was evaluated by measuring BRET between NanoLuc-TrkA and SnapTag-TrkA in HEK293T cells (**Fig. 7H**). Expression of increasing amounts of SnapTag-TrkA in HEK293T cells expressing a fixed amount of NanoLuc-TrkA produced a hyperbolic BRET signal, indicating oligomerization (**Fig. 7I**). NGF (30 nM) significantly enhanced this signal, consistent with agonist-evoked TrkA oligomerization. In cells expressing a fixed ratio of SnapTag-TrkA and NanoLuc-TrkA, NGF stimulated a concentration-dependent increase in BRET that was maximal after 5 min and sustained for at least 20 min (EC_{50} 15 nM; **Fig. 7J, Table 2**). While NGF had a lower potency with respect to dimerization than endocytosis, the kinetics of TrkA dimerization and endocytosis were similar. NRP1 overexpression enhanced TrkA oligomerization in response to higher NGF concentrations (>30 nM) (**Fig. 7K**). As dimerization is the first step in RTK activation, with well-established evidence for TrkA signaling from endosomes, these results provide a mechanistic insight into the role of NRP1 in controlling TrkA signaling and trafficking.

GIPC1 mediates NRP1/TrkA interactions and NGF-induced pain signaling

While NRP1 lacks intrinsic catalytic activity, the short cytoplasmic C-terminus interacts with G Alpha Interacting Protein (GAIP) Interacting Protein C-terminus 1 (GIPC1), or synectin, through a PDZ domain[32]. GIPC1 also interacts with the membrane-proximal regions of TrkA[33]. Thus, interaction with GIPC1 could underpin the co-receptor function of NRP1 in NGF/TrkA-evoked nociception. Analysis by RNAScope® *in situ* hybridization revealed that *Gipc1* mRNA is expressed by a large proportion of neurons in DRG from mouse (**Fig. 8A**). In humans, *Gipc1* was expressed by ~85-100% of neurons expressing *Ntrk1* mRNA (**Fig. 8B,C**).

GIPC1 is an intracellular adaptor protein that associates with receptors and channels to regulate their trafficking by interacting with the inwardly-directed myosin VI motor[34]. To determine whether GIPC1 is necessary for the TrkA chaperone function of NRP1, BRET between TrkA-Rluc8 and RGFP-CAAX was measured in HEK293T cells after GIPC1 knockdown. GIPC1 siRNA inhibited NRP1-induced plasma membrane expression of TrkA (**Fig. 8D**). In CAD.a cells, preincubation with a GIPC1 inhibitor (300 μM CR1023[35]) or a myosin VI inhibitor (50 μM TIP[36]) reduced NGF-induced TrkA trafficking to the plasma membrane (**Fig. 8E,F**). Similarly, GIPC1 siRNA inhibited the maximal response of NGF-induced ERK

signaling quantified using the downstream transcriptional reporter in CAD.a cells, with no effect on potency (**Fig. 8G**) (Ctrl siRNA pEC_{50} = 9.58 ± 0.03, GIPC1 siRNA pEC_{50} = 9.63 ± 0.09, n=5). GIPC1 siRNA also prevented NGF-induced action potential firing in mouse DRG nociceptors, determined by patch-clamp recordings (**Fig. 8H,I**). GIPC1 siRNA knockdown was confirmed at the mRNA level in HEK293T cells, CAD.a cells and DRG (**Supplementary Fig. 4A-C**).

To evaluate the role of GIPC1 in NGF-induced nociceptive behavior, GIPC1 or control siRNA was administered to mice by intrathecal (i.t.) injection 48 h before NGF intraplantar injection. In mice treated with control siRNA, NGF (50 ng/10 µl, i.pl.) caused mechanical allodynia and thermal hyperalgesia in the ipsilateral paw within 30 min for at least 24 h (**Fig. 8J,K**). GIPC1 siRNA prevented NGF-evoked mechanical allodynia and thermal hyperalgesia (**Fig 8J,K**). NGF or siRNA administration did not affect withdrawal responses of the contralateral paw to mechanical stimuli (**Fig. 8L**). The nociceptive role of GIPC1 was also investigated in a preclinical model of inflammatory pain in mice. Control or GIPC1 siRNA was administered (i.t.) 48 h after CFA (i.pl.). Knockdown of GIPC1 in DRG was confirmed using RNAScope® *in situ* hybridization (**Supplementary Fig. 4D**). While no effect was observed in the contralateral paw (**Fig. 8O**), GIPC1 siRNA inhibited CFA-induced mechanical allodynia and thermal hyperalgesia after 24 and 48 h (**Fig. 8M,N**). These results reveal a major role for GIPC1 in NGF-evoked pain.

DISCUSSION

NRP1 is a previously unrecognized co-receptor for NGF and TrkA that is necessary for NGF-induced nociception, demonstrated in both rodent and human tissue. NRP1 enhances NGF/TrkA signaling, since NRP1 inhibition abrogates the actions of NGF in intact mice, isolated nociceptors and cell lines, whereas NRP1 overexpression enhances TrkA signaling and trafficking. The results are consistent with evidence that NRP1 is a co-receptor for VEGF-mediated angiogenesis using pharmacological[24] or genetic[37] interventions. NRP1 is proposed to promote NGF/TrkA-mediated pain by at least two mechanisms: as a coreceptor that directly binds NGF, and as a chaperone that assembles a heterodimeric complex to enhance TrkA trafficking to and from the plasma membrane (**Fig. 9**).

The coreceptor function of NRP1 is supported by nanomolar affinity binding measurements between recombinant NRP1 and NGF. Molecular modeling indicates that the “CendR” motif of NGF remains exposed when interacting with TrkA[28], which could allow assembly of a trimeric NGF/TrkA/NRP1 complex. Despite the glycosylated nature of NRP1, indirect association of NGF with matrix components is unlikely to mediate NGF/NRP1 interactions as NGF does not directly interact with the extracellular matrix[38]. NRP1 potentiation of VEGFR2 signaling is linked to growth factor binding kinetics[26], similar to $p75^{NTR}$ potentiation of NGF/TrkA binding kinetics[39]. Further studies of the effects of NRP1 on NGF/TrkA binding kinetics are warranted. Since the NGF/$p75^{NTR}$ binding site[40] does not directly conflict with the proposed NGF/NRP1 binding site, a TrkA/$p75^{NTR}$/NRP1 complex could interact with NGF (*e.g.,* 2:1:1 stoichiometry), although there could be steric clashes between NRP1 and $p75^{NTR}$ for the same binding site on NGF.

The chaperone function of NRP1 is supported by BRET proximity assays showing that NRP1 and TrkA form a heteromeric complex and that NRP1 re-routes TrkA from the biosynthetic pathway to the plasma membrane and subsequently signaling endosomes. NRP1 colocalizes with TrkA to a greater extent than with VEGFR2[30]. Further studies are required to identify potential NRP1/TrkA interaction sites. TrkA redistribution to the cell surface would amplify exposure to extracellular NGF. Accordingly, NRP1 potentiates the effects of NGF on TrkA dimerization and endocytosis.

GIPC1 is a previously unrecognized mediator of pain. In the context of NGF/TrkA-evoked pain, GIPC1 interacts with TrkA[33] and NRP1[32], and could thus scaffold TrkA/NRP1 interactions to facilitate NGF signaling. GIPC1 disruption abrogates NRP1-stimulated translocation of TrkA to the plasma membrane and inhibits NGF-evoked endocytosis of TrkA and ERK signaling, in line with reports in other systems[41]. Considering GIPC1 regulates the trafficking of many receptors and ion channels, other mechanisms could mediate the antinociceptive effects of GIPC1 knockdown. Crucially, NRP1 and GIPC1 prominently colocalize with TrkA in human nociceptors where they could mediate NGF-induced pain. NRP1 may also serve as a co-receptor for NGF/TrkA signaling from adjacent cells, akin to VEGFR2 modulation from both ‘cis’ (same cell) and ‘trans’ (neighboring cell) forms[42]. This intercellular signaling may underlie functional interactions between nociceptors and adjacent cells that contribute to pain (*e.g.,* Schwann cells).

Electrophysiological and behavioral studies support the essential role of NRP1 and GIPC1 in NGF-induced pain. Two chemically distinct small molecule NRP1 inhibitors and a mAb against the b1 domain of NRP1 prevent NGF-evoked mechanical allodynia and thermal hyperalgesia in mice, providing confidence in specificity. GIPC1 siRNA similarly prevents NGF-induced nociception. NRP1 and GIPC1 inhibition blunts inflammatory nociception, indicating that NRP1 and GIPC1 mediate the nociceptive action of endogenous and exogenous NGF. NRP1 is necessary for NGF-induced action potential firing in both mouse and human DRG neurons, supporting human translation. NRP1 is required for the proximal events of NGF-evoked nociception (TrkA dimerization, phosphorylation, channel activation), as well as sustained components of pain signaling (ERK activation, transcription), both of which mediate nociceptor sensitization[43]. Although TrkA is the principal pronociceptive NGF receptor, $p75^{NTR}$ also contributes to NGF-induced pain[44] and the effect of NRP1 on $p75^{NTR}$ warrants further study. NGF/TrkA signalosomes transported from nerve terminals to the soma have been studied in compartmentalized sympathetic[45] and DRG[46] neuronal cultures, where retrograde NGF/TrkA signaling controls gene expression during the development of sympathetic neurons. Whether endosomal signaling of NGF/TrkA contributes to pain, as observed with G protein-coupled receptors[47], deserves further attention. Additional studies are also required to determine whether NRP1 is necessary for NGF-induced expression of pronociceptive transmitters and channels, and whether NRP1 mediates the neurotrophic actions of NGF. NRP1 interacts with semaphorins[19] as a co-receptor for plexins, which regulate TrkA retrograde signaling[48]. Accordingly, NRP1 is also highly expressed in axonal growth cones[49], as well as small diameter NGF-responsive DRG neurons during neurite sprouting[50]. Thus, disruption of NRP1 could affect NGF-induced neuronal growth, with implications for the neuroplasticity associated with chronic pain.

NGF is implicated in neuropathic, surgical and cancer pain[5], highlighting the need to study contributions of NRP1 and GIPC1 across pain pathologies. Upregulation of NRP1 and GIPC1 in painful conditions, such as cancer, could amplify NGF/TrkA signaling. Changes in NRP1 expression have been reported in migraine patients[51]; though presumed to link to vasculature changes through VEGF signaling, this could mediate migraine pain through NGF[52]. Given the key role of NGF/TrkA in pain and the challenge of developing TrkA-selective antagonists, NGF mAbs were prioritized for the treatment of pain. While NGF mAbs provided beneficial pain relief to patients with arthritis, rapidly progressing osteoarthritis observed in some patients has precluded FDA approval[12]. It is unknown whether NRP1 contributes to NGF signaling in bone tissue, considering its expression on osteoblasts[53]. Similar to the development of partial or biased drugs that 'fine-tune' G protein-coupled receptor signaling to reduce adverse effects, targeting NGF signaling *via* co-receptors/scaffolds could be advantageous for treating patients that suffer from chronic pain without the side effects of systemic NGF sequestration. Identifying the antinociceptive efficacy of vesencumab highlights an opportunity to repurpose a biologic developed for cancer for the treatment of chronic pain, facilitating the development of a non-opioid therapeutic targeting NGF signaling through this novel co-receptor.

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AUTHOR CONTRIBUTIONS

C.J.P., R.T., K.G., M.R-S., A.C-R., L.M., A-M.M., S.P-M., H.H., A.R.B.T., S.D., B.L.S., M.K., R.K., N.W.B. designed and conducted the experiments, performed the analysis, discussed the results, and wrote the paper.

COMPETING INTERESTS

Nigel W. Bunnett is a founding scientist of Endosome Therapeutics and pH-ARM Therapeutics. Research in his laboratory is funded in part by Takeda Therapeutics.

MATERIALS AND METHODS

Compounds. Recombinant βNGF (R&D Systems or Alomone Labs) and recombinant NRP1 (R&D Systems) were reconstituted according to manufacturer's instructions. GIPC1 inhibitors (CR1023 or negative control) were from Genscript. Myosin VI inhibitor (2,4,6-triiodophenol) was from Thermo Scientific Chemicals. Unless stated otherwise, *in vitro* studies used human NGF, whereas *ex vivo* and *in vivo* studies used murine NGF.

cDNAs. SnapTag-TrkA was cloned with a GSSGAIA spacer and an N-terminal interleukin-6 signal sequence (IL-6-SnapTag-TrkA). IL-6-NanoLuc-TrkA, IL-6-NanoLuc-p75^{NTR} and TrkA-HA-Rluc8 were cloned using Gibson assembly. HiBiT, a short fragment of NanoLuc[54], was cloned on the N-terminus of VEGF$_{165}$a or C-terminus of βNGF with a GSSG linker. HaloTag-NRP1, SnapTag-NRP1 and NanoLuc-NRP1 were from Dr. Laura Kilpatrick and Prof. Stephen Hill (University of Nottingham). SnapTag-CALCRL was from Alex Thomsen (New York University). RGFP-CAAX (prenylation CAAX box of KRas), tdRGFP-Rab5a, tdRGFP-Rab4a and tdRGFP-Giantin were from M. Bouvier (Université de Montréal). Nuclear and cytosolic EKAR (CFP/YFP) FRET biosensors were from Addgene.

Cell Culture. HEK293T cells were grown in complete Dulbecco's modified Eagle's medium GlutaMAX™ supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin-streptomycin. CAD.a cells were grown in RPMI 1640 containing 4% FBS, 8% horse serum and 100 U/mL penicillin-streptomycin.

siRNAs. ONTARGETplus siRNA targeting human GIPC1 (L-019997-00-0010), murine GIPC1 (L-062534-00-0005) or nontargeting control (Ctrl) siRNA (D-001810-10-05) were from Dharmacon (**Supplementary Table 3**).

Transfection of cell lines. HEK293T cells were transfected using polyethylenimine (PEI, Polysciences; 1:6 DNA:PEI) diluted in a 150 mM NaCl solution. CAD.a cells were transfected with Lipofectamine 3000 (according to manufacturer's instructions) diluted in serum-free DMEM.

Animals. Experiments were in accordance with the guidelines recommended by the National Institute of Health, the International Association for the study of Pain, the National Centre for the Replacement, Refinement and Reduction of Animals in Research (ARRIVE) guidelines, and were approved by the New York University Institutional Animal Care and Use Committee. Male C57BL/6 mice (8-10 weeks, Charles River) were housed four per cage at 22 ± 0.5°C under a controlled 14/10 h light/dark cycle with free access to food and water. Mice were randomly assigned to experimental groups; group size was based on previous similar studies. Investigators were blind to treatments.

Molecular docking. The predicted structure of full-length human NRP1 (UniProt O14786) was obtained from AlphaFold. In the downloaded model, both the transmembrane region and the MAM domains were folded against the a1a2b1b2 globular domains; therefore, domains were removed from the model prior to docking, leaving only the a1a2b1b2 domains. The AlphaFold predicted structure of full-length NGF (UniProt P01138) or the NGF/NRP1 complex was modeled using PIPER[55] from within the Schrödinger Biologics Suite (Release 2022-1). For PIPER, the NMR structure of the human NGF dimer[56] contains a C-terminal Arg118. All 20 NMR models were examined and one with the C-terminal residues in an extended conformation was selected. Initial PIPER docking analysis revealed that the orientation of the a1 domain potentially inhibited access of the NGF dimer to the b1 domain. Thus, the position of the NRP1 a1 domain was modified to match that observed in the structure of mouse NRP1[21]. Model rebuilding was performed in COOT with Ramachandran restraints. Models were prepared using the Schrödinger Suite and PIPER docking was repeated using standard mode with a single attractive distance restraint that Arg118 (NGF chain A) and Asp320 (NRP1) sidechains be within 2-4 Å. Other settings were left at default values: standard mode, a maximum of 70,000 ligand rotations to probe, a maximum of 30 poses returned, and output poses were refined. All of the 30 returned clusters had Arg118 in or near to the b1 pocket. Only three clusters had the Arg118 side-chain terminal nitrogens oriented toward the terminal oxygens of Asp320. The largest of these clusters (117 members, PIPER pose energy = 634 kcal/mol, PIPER pose score -66 kcal/mol) was selected for visualization of potential interactions.

Microscale thermophoresis. Purified NRP1-His (Bio-Techne, 3870-N1) was fluorescently labeled using the Monolith Protein Labeling kit RED-Tris-NTA (NanoTemper). 200 nM of NRP-His in Tris 40 mM, NaCl 250 mM and DTT 1 mM was mixed with NT-647-His-labeling dye (30 min, RT). Labeled NRP was centrifugated (15,000 g, 10 min, 4°C). NRP1-His (15 nM) was mixed with increasing concentrations of unlabeled NGF (10^{-10} M to 10^{-5} M) in PBS (10 min, RT). Thermophoresis was measured using a Monolith

446 NT.115 (NanoTemper), using premium MST capillaries at 100% MST power. Data were analyzed with MO
447 Affinity Analysis software using the K_d model.

448 **Binding using HiBiT-BRET**. HEK293T cells were transfected with SnapTag-NRP1 or SnapTag-NRP1
449 Y297A (3 µg/10 cm dish) using PEI. After 24 h, cells were transferred to 96-well plates. The next day, cells
450 were incubated with SNAP-Surface® Alexa Fluor® 488 (0.25 µM, 1 h, 37ºC), then washed in HBSS/0.1%
451 BSA. Separately, HEK293T cells were transfected with IL-6-HiBiT-VEGF$_{165}$a (3 µg/10 cm dish) or IL-6-
452 NGF-HiBiT (5 µg/dish). After 48 h, medium was replaced with HBSS/0.1% BSA to collect secreted growth
453 factor (2 h, 37ºC). Supernatant was diluted (1:2) in HBSS/0.1% BSA and incubated with recombinant
454 HaloTag-LgBiT protein and furimazine (1:50 final dilution for both; Promega Corporation). NRP1-expressing
455 cells were pre-incubated with vehicle or unlabeled VEGF$_{165}$a (10 nM, 30 min), followed by supernatant with
456 furimazine (1:2, 15 min, 37ºC). Luminescence and fluorescence emissions were recorded (Synergy Neo2,
457 Agilent BioTek; BRET1 donor 460 ± 40 nm, acceptor 540 ± 25 nm).

458 **Collection of mouse tissue.** Mice were anesthetized (5% isoflurane) and perfused through the ascending
459 aorta with PBS and then 4% paraformaldehyde in PBS. DRG (vertebrae L4-L5) were removed, fixed in 4%
460 paraformaldehyde in PBS (1 h, 4°C), cryoprotected in 30% sucrose (24 h, 4°C), and embedded in Optimal
461 Cutting Temperature compound (Tissue Tek). Frozen sections (10-12 µm) were mounted, dried (15 min)
462 and stored (-20°C).

463 **Collection of human tissue.** For RNAScope® studies, the collection of DRG from deidentified organ
464 donors was reviewed by the Institutional Review Board of the University of Cincinnati (#00003152, Study
465 ID 2015-5302) and deemed to be human subjects exempt. Donor information has been provided[57]. DRG
466 (L4-L5) were collected in the operating room within 90 min of aortic cross clamp and after removal of vital
467 organs. DRG were placed in N-methyl-D-glucamine-artificial cerebrospinal fluid (4°C), dissected and
468 immersion fixed in 4% paraformaldehyde in PBS (overnight, 4°C), cryoprotected in 30% sucrose (24 h,
469 4°C), and embedded in Optimal Cutting Temperature compound. Frozen sections (14-18 µm) were
470 mounted, dried (15 min) and stored (-80°C).

471 **Immunofluorescence.** Mouse tissue was blocked (2% bovine serum albumin, BSA, 0.2% Triton X-100 in
472 PBS; 1 h, room temperature (RT)). Sections were incubated (4ºC, overnight) with monoclonal rabbit anti-
473 TrkA (1:50; Millipore-Sigma, SU0354), and monoclonal mouse anti-NRP1 (1:200; Santa Cruz, A-12) or
474 monoclonal human anti-NRP1 vesencumab (1:500; Invitrogen, MA5-41940). Slides were washed and
475 incubated with donkey anti-rabbit Alexa Fluor® 488 (1:1000) and either donkey anti-mouse or anti-human
476 Alexa Fluor® 647 (1:1000, 1 h, RT). Slides were incubated with DAPI (1 µg/ml, 5 min) and mounted
477 (ProLong® Gold Antifade). Sections were imaged using a Leica SP8 confocal microscope with HCX PL
478 APO 10x air objective (Leica Microsystems).

479 **RNAScope® *in situ* hybridization in mouse and human tissue.** RNAScope Multiplex Fluorescent
480 Reagent v2 Assay (Advanced Cell Diagnostics Inc.) was carried out for fresh-frozen tissue as
481 recommended by the manufacturer, except for omission of the initial on-slide fixation step with mouse
482 tissue. Probes to Mm-Nrp1(#471621-C3), Mm-Ntrk1 (#435791-C2), Mm-Gipc1 (#1232971-C1), Hs-Nrp1
483 (#424361-C1) Hs-Ntrk1 (#402631-C2) and Hs-Gipc1 (#1232961-C3) were used. Mouse tissue was
484 incubated with Opal 620 (1:1000, FP1495001KT, Akoya Biosciences) and Opal 520 (FP1487001KT)
485 reagents for fluorescence detection. For human tissue, Tyramide Signal Amplification Plus Cyanine 3
486 (1:1500) and Cyanine 5 (1:1000) were used to detect the hybridized signals. To detect neurons in mouse
487 tissue, hybridized slides were blocked and incubated with guinea pig anti-NeuN antibody (1:500, overnight,
488 4°C; EMD Millipore, N90;). Slides were washed and incubated with goat anti-guinea pig Alexa Fluor® 647
489 (1:1000, 45 min, RT). Alternatively, neurons were detected using Nissl staining. Slides were washed,
490 incubated with DAPI and mounted. For mouse tissue, sections were imaged using a Leica SP8 confocal
491 microscope with HCX PL APO 40x (NA 1.30) oil objective. The percentage of hybridized positive neurons
492 were quantified and normalized by the total number of neurons. For human tissue, images were captured
493 as stitched Z stacks using Keyence BZ-X800E fluorescence microscope. Cells positive for Ntrk1, Nrp1 and
494 Gipc1 were counted to mark positive cells, where 10+ fluorescent puncta were considered to be positive.

495 **Human DRG cultures.** Human DRG suspension cells were obtained from AnaBios Corporation. Cells were
496 recovered with a gentle centrifugation at room temperature (3 min) and resuspension with DMEM/F12
497 media containing 10% horse serum, 1% penicillin-streptomycin, 25 ng/ml hNGF and 25 ng/ml GDNF. Cells
498 were seeded on poly-D-lysine (0.1 mg/ml) and laminin (1 mg/ml)-coated 12-mm glass coverslips. Half of
499 the culture medium was replaced with fresh medium every 3 days. All cultures used within 96 h.

Mouse DRG cultures. C57BL/6 mice (~3-4 weeks) were anaesthetized (5% isoflurane) and decapitated. Thoracic and lumbar DRG were excised, then ganglia dissociated by collagenase type I (1 mg/ml) and neutral protease (0.62 mg/ml; 45 min, 37°C) under gentle agitation. Dissociated cells were pelleted and resuspended in DMEM containing 10% FBS and 1% penicillin-streptomycin. For siRNA transfection, cells were resuspended in Nucleofector® transfection reagent containing GIPC1 or Ctrl siRNA (500 nM) and a plasmid encoding the enhanced green fluorescent protein (eGFP, 1 µg). Cells were electroporated using Amaxa Biosystem (Lonza) with protocol O-003. Neurons were plated on glass coverslips pretreated with poly-D-lysine (0.1 mg/ml) and laminin (0.01 mg/ml; 30 min). Neurons were used within 48 h and siRNA transfection verified by eGFP fluorescence.

Whole-cell patch-clamp recordings of evoked action potentials in mouse DRG neurons. For current-clamp recordings, the external solution contained (mM): 154 NaCl, 5.6 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 D-Glucose, and 8 HEPES (pH 7.4 adjusted with KOH, and mOsm/L= 300). The internal solution comprised (mM): 137 KCl, 10 NaCl, 1 $MgCl_2$, 1 EGTA, and 10 HEPES (pH 7.3 adjusted with KOH, and mOsm/L= 277). Recordings of action potentials were made at RT in whole-cell patch clamp configuration and current-clamp mode. DRG neurons with a resting membrane potential (RMP) more hyperpolarized than −40 mV, stable baseline recordings, and evoked spikes that overshot 0 mV were used for experiments and analysis. The action potentials were evoked by a ramp pulse from 0-1000 pA in 1 sec for human DRG, or 0-250 pA in 1 sec for mouse DRG. Rheobase was measured by injecting currents from 0 pA with an increment of 50 pA in 100 ms (human) or 10 pA in 50 ms (mouse). Analyses were performed by using Fitmaster software (HEKA) and Origin 9.0 software (OriginLab).

Whole-cell patch-clamp recordings of Ca^{2+} and Na^{+} currents in mouse DRG neurons. Currents were recorded using an EPC 10 Amplifier-HEKA (HEKA Elektronik) with Patchmaster software. DRG neurons were preincubated with EG00229 (30 µM, 30 min) or vehicle (0.1% DMSO) and then challenged with NGF (50 nM, 30 min). Ca^{2+} currents (ICa^{2+}) and Na^{+} (INa^{+}) currents were recorded as described[17,18]. In brief, peak Ca^{2+} current was acquired by applying 200-millisecond voltage steps from −70 to +70 mV in 10-mV increments from a holding potential of −90 mV to obtain the current-voltage (I-V) relation. Peak Na^{+} current was acquired by applying 150-millisecond voltage steps from −70 to +60 mV in 5-mV increments from a holding potential of −60 mV to obtain the current-voltage (I-V) relation. Capacitive artifacts were fully compensated, and series resistance was compensated by ∼70 %. Recordings made from cells with greater than a 20% shift in series resistance compensation error were excluded from the analysis. All recordings were made at RT (∼23 °C).

NGF-evoked nociception. Mouse NGF (50 ng/10 µl) was administered by i.pl. injection into the right hindpaw of mice. EG00229 (MedChemExpress, HY-10799), compound 5[27], a human monoclonal anti-NRP1 antibody (Vesencumab, Invitrogen, MA5-41940), or IgG control (Ctrl, ThermoFisher 02-6502) was administered (i.pl.) concomitantly with NGF.

Inflammatory pain. CFA (1 mg/ml) or vehicle (0.9% NaCl) was administered (10 µl, i.pl.) into the right hindpaw of sedated mice (2% isoflurane). NRP1 inhibitors were injected 48 h after CFA.

Intrathecal administration of siRNA. Mouse GIPC1 or Ctrl siRNA (1.25 µg) was mixed with *in vivo*-jetPEI transfection reagent (8:1 PEI:DNA; Polyplus, 201-50G) and administered to conscious mice (5 µl, i.t., L4-L5), 48 h before NGF (i.pl.) or 48 h after CFA (i.pl.). *Gipc1* mRNA in DRG (L4-L5) was analyzed by RNAScope® *in situ* hybridization, 48 h after siRNA injection.

Nociception assays. Investigators were blinded to treatments. Mechanical allodynia was assessed by measuring hindpaw withdrawal response to VFF stimulation using the up-and-down method[57]. The Hargreaves apparatus was used to evaluate hypersensitivity to heat (Ugo Basile)[57].

Phosphorylated TrkA assays. Acutely dispersed mouse DRG neurons were plated onto coverslips pre-coated with Cell-Tak (30 min, 37°C; CB-40240) in DMEM containing 10% FBS. After 18 h, neurons were incubated with a monoclonal NGF antibody (1:500, 4 h, 37°C; MA5-41968) in serum-free DMEM to reduce interference from endogenous NGF. Neurons were washed in HBSS/0.1% BSA, pre-incubated with EG00229 (30 µM, 30 min) or control, then stimulated with vehicle or murine NGF (100 nM, 15 min, 37°C). Neurons were fixed with 4% paraformaldehyde in PBS (20 min, 4°C), blocked (2% BSA, 0.2% Triton-X-100 in PBS), and incubated with a rabbit mAb to phosphorylated TrkA Y785/TrkB Y816 (1:500, overnight, 4°C; Cell Signaling, 4168). Neurons were washed in PBS and incubated with donkey anti-rabbit Alexa Fluor® 488 (1:1:000, 1 h, RT). Slides were incubated with DAPI, mounted then imaged using a Leica SP8 confocal microscope with HCX PL APO 40x (NA 1.30) oil objective. Mean fluorescence intensity was quantified from

554 regions of interest drawn using a phase contrast image with the maximum projection from four Z planes
555 (0.5 µm thickness). Images were quantified from 34-44 individual cells across three independent
556 experiments using separate mice.

557 **ERK activity FRET biosensor assays.** CAD.a cells were serum-starved and transfected with EKAR FRET
558 biosensors (300 ng/well) and human SnapTag-TrkA (150 ng/well). HEK293T-FLPN cells were transfected
559 with FRET biosensors (1 µg/10 cm dish), SnapTag-TrkA (1 µg), and pcDNA3.1 or HaloTag-NRP1 (2 µg).
560 After 24 h, cells were transferred to 96-well plates and serum-starved overnight. On the day of the assay,
561 cells were washed and incubated in Hanks' buffered saline solution (HBSS, pH 7.4) containing 0.1% BSA.
562 After 30 min (37°C), FRET was measured at 60 s intervals (CLARIOstar, BMG Labtech). After 5 baseline
563 reads, cells were stimulated with NGF (0.1 pM-10 nM) or phorbol 12,13-dibutyrate (PDBu, 10 µM). **Δ**FRET
564 represents the ratio (YFP/CFP), minus the mean from 5 initial reads and baseline-corrected to vehicle. Area
565 under the curve (AUC) was determined for each replicate.

566 **ERK transcriptional assays.** HEK293T-FLPN cells were transfected with SRE-Luc2P (3 µg/10 cm dish),
567 SnapTag-TrkA (1 µg) and either pcDNA3.1 or HaloTag-NRP1 (2 µg). After 24 h, cells were transferred to
568 96-well plates. CAD.a cells were transfected with SRE-Luc2P (250 ng/well, 96-well plate), SnapTag-TrkA
569 (150 ng/well) and 100 nM siRNA. After 24 h, cells were serum-starved overnight. Cells were then incubated
570 in HBSS/0.1% BSA and stimulated with NGF (1 pM-10 nM) or PDBu (10 µM). After 5 h stimulation (37ºC),
571 cells were incubated with luciferin (1:2) and lysis buffer (1:5). After 5 min to enable the substrate reaction,
572 luminescence emissions were recorded (CLARIOstar).

573 **Live imaging.** HEK293T cells were transfected with SnapTag-TrkA (1 µg/10cm dish), HaloTag-NRP1 (2
574 µg) or both TrkA and NRP1. After 24 h, cells were transferred to 35 mm dishes. CAD.a cells were seeded
575 in 35 mm dishes, serum-starved for 24 h, and then transfected with SnapTag-TrkA (250 µg/35 mm),
576 HaloTag-NRP1 (500 µg) or both. The next day, proteins were labeled with SNAP-Surface® Alexa Fluor®
577 488 and membrane-impermeant HaloTag® Alexa Fluor® 660 (0.5 µM, 30 min, 37ºC). Cells were washed
578 in HBSS and imaged on the Leica SP8 confocal.

579 **Receptor-receptor BRET**. For endpoint studies, HEK293T cells were transfected with a fixed
580 concentration of NanoLuc-NRP1, NanoLuc-p75^{NTR} or NanoLuc-TrkA (10 ng/well, 96-well plate) and
581 increasing concentrations of SnapTag-TrkA or SnapTag-CALCRL (0-50 ng/well). For kinetic studies, cells
582 were transfected with NanoLuc-TrkA (10 ng/well), SnapTag-TrkA (25 ng/well) and either pcDNA3.1 or
583 HaloTag-NRP1 (50 ng/well). After 48 h, cells were incubated with SNAP-Surface® Alexa Fluor® 488 (0.5
584 µM, 30 min, 37ºC), washed in HBSS before fluorescence emissions were measured (Synergy Neo2). In
585 some conditions, cells were incubated with NGF (30 nM, 30 min, 37ºC) or vehicle. Cells were then incubated
586 with furimazine (10 µM, 5 min, Promega). For kinetic studies, cells were stimulated with increasing NGF
587 concentrations (1 pM-100 nM) following 5 baseline reads. Luminescence and fluorescence emissions were
588 quantified (Synergy Neo2; BRET1 filter).

589 **Quantifying SNAP-Surface® Alexa Fluor® 488**. HEK293T cells were transfected with SnapTag-TrkA (25
590 ng/well, 96-well plate) and pcDNA3.1 or HaloTag-NRP1 (50 ng/well). CAD.a cells were transfected with
591 SnapTag-TrkA (150 ng/well) and either pcDNA3.1 or HaloTag-NRP1 (300 ng/well). Cells were labeled using
592 SNAP-Surface® Alexa Fluor® 488 and HaloTag® Alexa Fluor® 660 (0.5 µM, 30 min, 37ºC), washed in
593 HBSS/0.1% BSA and fluorescence emissions were recorded (CLARIOstar).

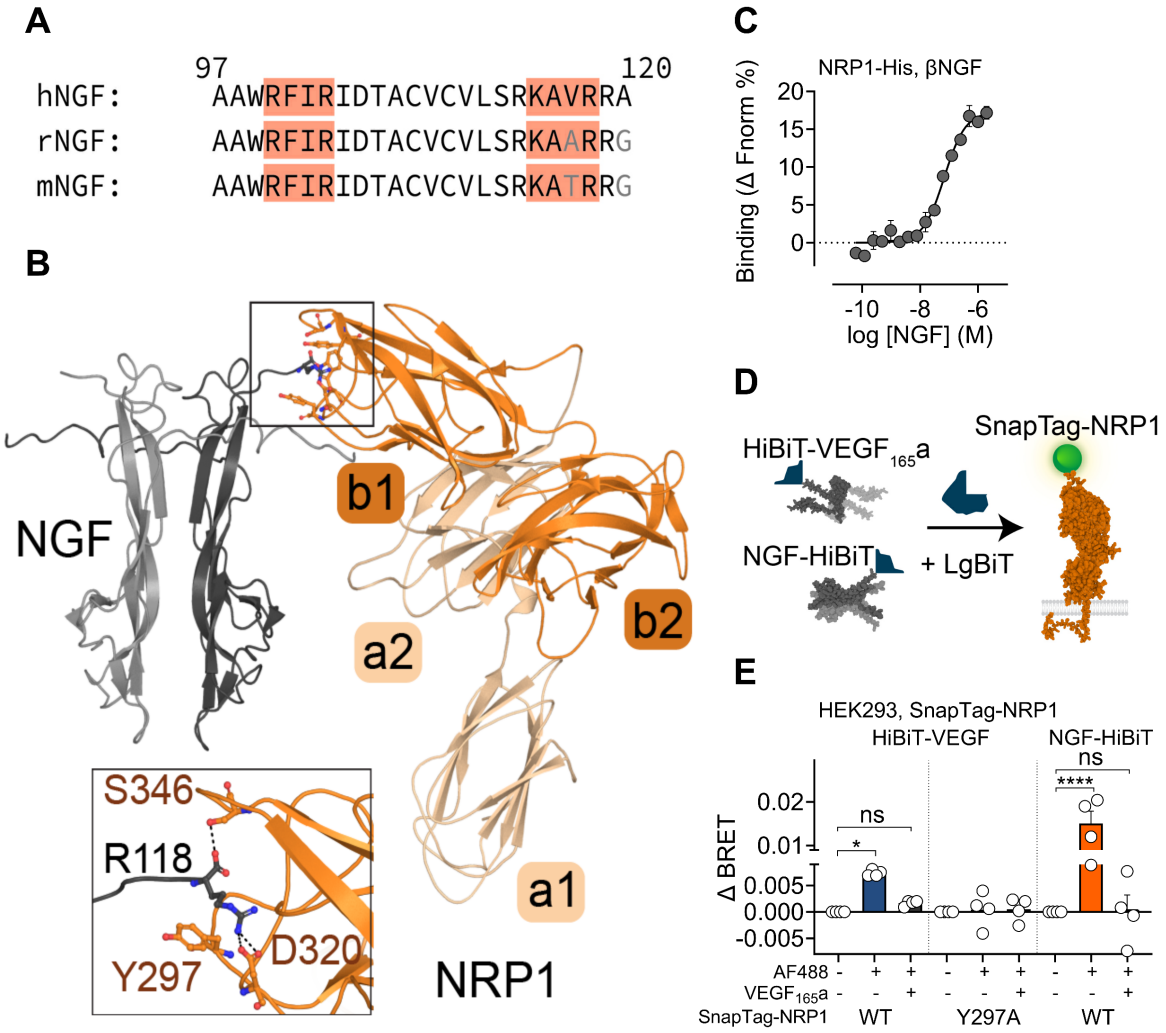
594 **BRET trafficking.** HEK293T cells were transfected with TrkA-HA-Rluc8 (15 ng/well, 96-well plate) and a
595 fluorescent marker (15 ng/well) for the plasma membrane (RGFP-CAAX), early endosome (tdRGFP-
596 Rab5a), recycling endosome (tdRGFP-Rab4a) or cis-Golgi apparatus (tdRGFP-Giantin). In some
597 conditions, cells were co-transfected with pcDNA3.1 or HaloTag-NRP1 (30 ng/well), or 100 nM siRNA.
598 Alternatively, CAD.a cells were transfected with TrkA-HA-Rluc8 (125 ng/well) in the absence or presence
599 of HaloTag-NRP1 (250 ng/well), as well as RGFP-CAAX, tdRGFP-Rab5a, tdRGFP-Rab4a or tdRGFP-
600 Giantin (150 ng/well). After 48 h, cells washed in HBSS/0.1% BSA were pre-incubated (30 min, 37ºC) with
601 hypertonic sucrose (0.45 M), Pitstop 2 (30 µM; Abcam), an inhibitor of GIPC1 (CR1023, 300 µM) or its
602 negative control (CR2055), or an inhibitor of myosin VI (TIP, 50 µM). Cells were incubated with
603 coelenterazine purple (25 µg/ml, 10 min, Nanolight). For kinetic studies, 5 baseline reads were measured,
604 then cells were stimulated with NGF (1 pM-100 nM), using 30 nM NGF for endocytic inhibitors. BRET ratios
605 were recorded every 45 s (Synergy Neo2; BRET2 filter: donor 410 ± 80 nm, acceptor 515 ± 30 nm). **Δ**BRET
606 represents BRET in the presence of agonist, minus the vehicle BRET over time. For endpoint studies

607 lacking agonist stimulation, BRET ratios were baseline-corrected to TrkA-HA-Rluc8 expression alone
608 (100%).

609 **Quantitative PCR (qPCR)**. RNA was isolated from cells or snap-frozen tissues using Direct-zol RNA
610 MiniPrep Kit (Zymo Research, R2050). cDNA was prepared using MultiScribe Reverse Transcriptase
611 (Thermo Fisher, 4311235). cDNA was amplified for 40 cycles by qRT-PCR using the QuantStudio 3 Real-
612 Time PCR System and TaqMan Fast Advanced PCR Mastermix (4444556). Primers to hTrkA
613 (Hs01021011_m1), mTrkA (Mm01219406_m1), hNgfr (Hs00609976_m1), mNgfr (Mm00446296_m1),
614 hGIPC1 (Hs00991802_m1), mGIPC1 (Mm00457561_m1), or GAPDH (Mn99999915_g1) were used. The
615 relative abundance of mRNA was calculated as described[57].

616 **Data analysis**. Data presented as mean ± SEM using GraphPad Prism (8.0). Images processed using
617 ImageJ. Illustrations were produced using Adobe Illustrator, Mol*Viewer or BioRender. Concentration-
618 response data fit using non-linear regression analysis as log *vs.* response (three parameters) to
619 determine EC_{50} values. Differences were assessed using paired or unpaired t-test for two comparisons,
620 and 1- or 2-way ANOVA and Tukey's, Dunnett's or Šidák's post-hoc test for multiple comparisons. $P<0.05$
621 was considered significant at the 95% confidence level.

622 **FIGURES & FIGURE LEGENDS**



623

624 **Figure 1. NGF directly interacts with NRP1.** (**A**) Carboxy-terminal sequences of NGF, highlighting
625 prospective CendR motifs (R/KxxR/K). Amino acids numbered according to the mature βNGF sequence
626 (1-120 equivalent to proNGF 122-241). *h, Homo sapiens; r, Rattus norvegicus; m, Mus musculus*. (**B**)
627 Prospective binding site between human βNGF and NRP1 modelled using protein docking PIPER software.
628 (**C**) Interaction between purified His-tagged human NRP1 (residues 22-644) and unlabeled human beta-
629 NGF using MST. Data from 4 independent experiments. Mean±SEM. (**D-E**) Bioluminescence resonance
630 energy transfer (BRET) to measure proximity (<10 nm) at full-length NRP1 in living HEK293T cells at 37ºC.
631 Supernatant was collected from cells secreting growth factor ($VEGF_{165}a$ or NGF) tagged with HiBiT, a small
632 portion of nanoluciferase with high affinity. When reconstituted with recombinant LgBiT and luciferase
633 substrate, this acts as a bioluminescent donor for SnapTag-NRP1 labeled with SNAPTag-Alexa Fluor® 488
634 (AF488). BRET was compared to negative control (HiBiT/LgBiT only lacking AF488). Cells were pre-
635 incubated with vehicle or 10 nM unlabeled $VEGF_{165}a$ (30 min), followed by luminescent growth factor (15
636 min, 37ºC). BRET with HiBiT-$VEGF_{165}a$ was measured between HiBiT-$VEGF_{165}a$ and either SnapTag-
637 NRP1 (WT) or the known $VEGF_{165}a$ binding-dead mutant (Y297A), as well as HiBiT-tagged NGF (**E**). Data
638 from 4 independent experiments with triplicate wells. F. 1-way ANOVA, Šídák's multiple comparisons.
639 *P<0.05, **P<0.01, *** P<0.001, ****P<0.0001.

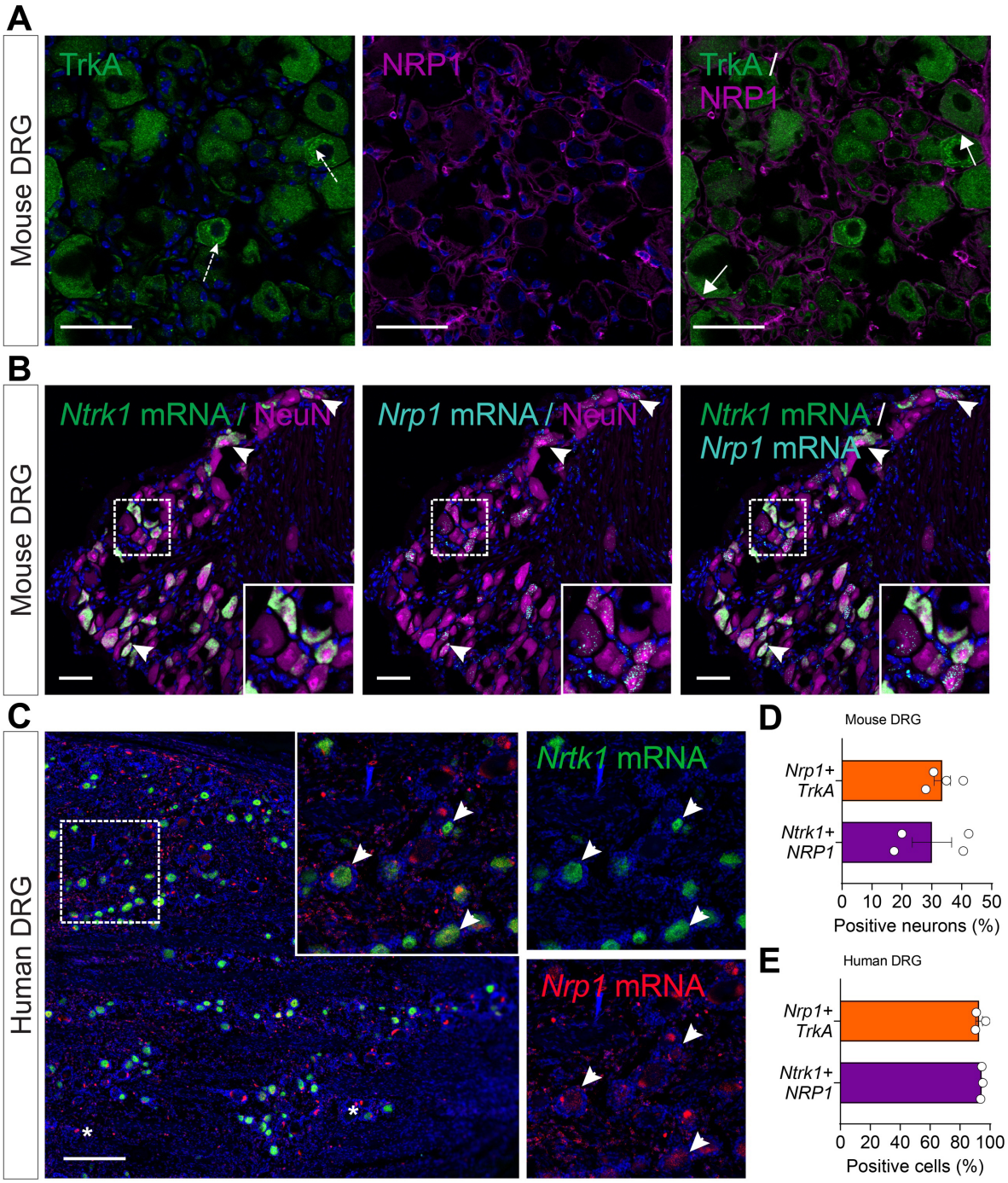


Figure 2. *Ntrk1*/TrkA and *Nrp1*/NRP1 are coexpressed in DRG. (**A**) TrkA and NRP1 immunofluorescence in mouse DRG, showing subcellular TrkA (dashed arrows), and cells coexpressing TrkA and NRP1 (solid arrows). Scale bar, 50 µm. (**B**) RNAScope® localization of *Ntrk1* and *Nrp1* mRNA in mouse DRG, showing mRNA expression in the same neuron using NeuN immunofluorescence (arrowheads). Scale bar, 500 µm. (**C**) RNAScope® localization of *Ntrk1* and *Nrp1* mRNA in human DRG, showing mRNA expression in the same cell (arrowheads). * Denotes fluorescence due to lipofuscin (*). Nuclei shown in blue. (**D, E**) Quantified co-expression of *Ntrk1* and *Nrp1* mRNA in mouse DRG (**D**) or human DRG (**E**). Representative images, n=4-5 mice; n=3 human sections. Hybridized positive cells (%) were normalized to the total number of cells.

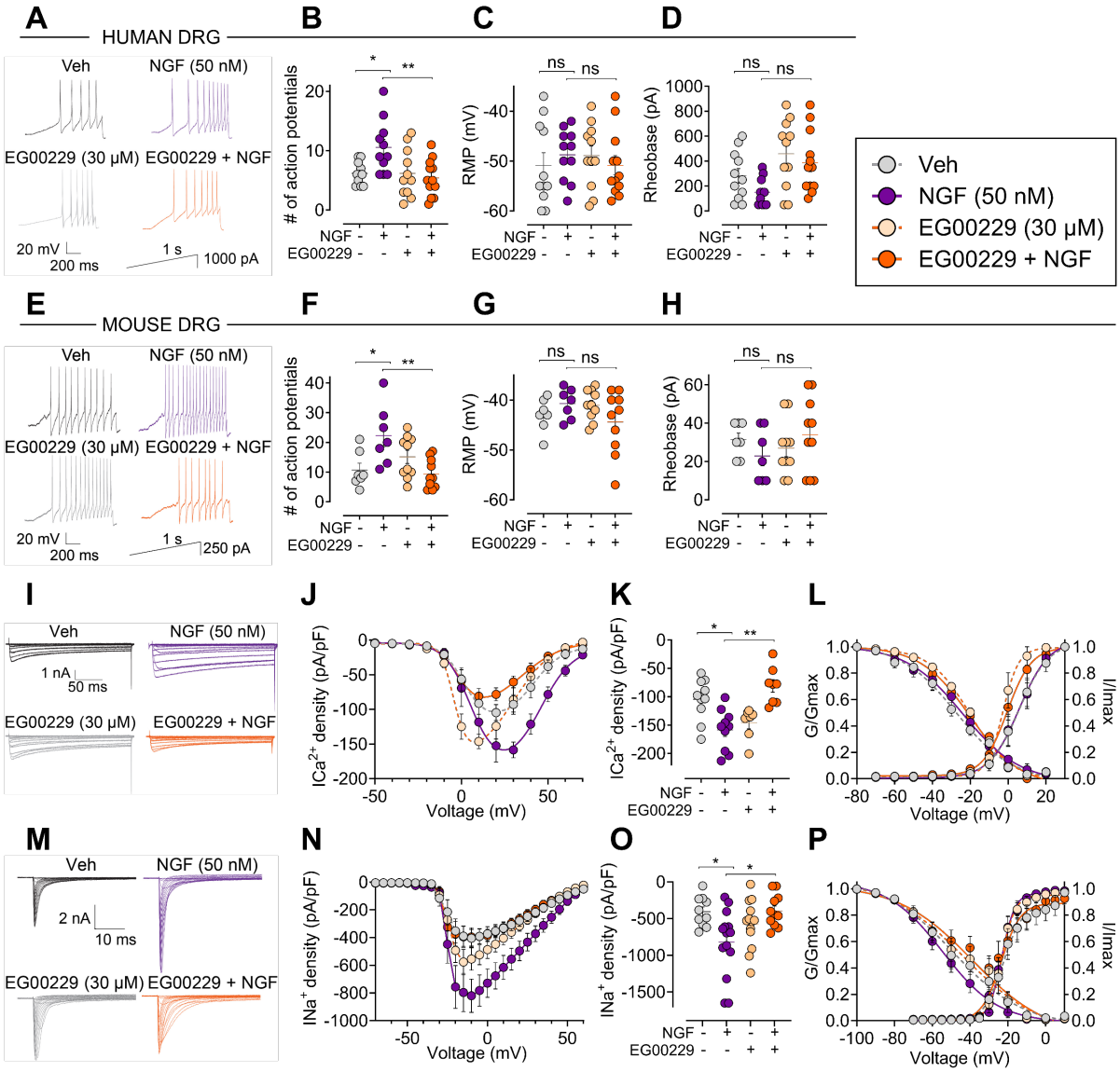


Figure 3. NRP1 inhibition prevents NGF-mediated increases in neuronal firing and ion channel currents. Effect of NRP1 inhibitor EG00229 (30 μM, 30 min) on responses of dissociated human (A-D) or mouse DRG neurons (**E-P**) to NGF (50 nM, 30 min). (**A-B,E-F**) Representative action potential firing evoked by a depolarizing ramp stimulus (**A,E**), with summary of the number of evoked action potentials (**B,F**). N=7-10 cells. (**C-D,G-H**) Resting membrane potential (**C,G,** millivolts, mV) and rheobase (**D,H**, picoamperes, pA). (**E-H**) Representative family of Ca^{2+} current traces recorded from small diameter DRG neurons in response to depolarization steps from -70 to +70 mV from a holding potential of -90 mV (**E**), with double Boltzmann fits for current density-voltage curve (**F**), summary of peak calcium current densities (**G**; pA/pF) and Boltzmann fits for voltage-dependence of activation and inactivation (**H**). N=7-10 cells. (**I-L**) Representative family of Na^{+} current traces, where currents were evoked by 150 ms pulses between −70 and +60 mV, with double Boltzmann fits for current density-voltage curve (**J**), summary of peak sodium current densities (**K**, pA/pF) and Boltzmann fits for voltage-dependence of activation and inactivation (**L**). N=10-14 cells. Mean±SEM. *P<0.05, ** P<0.01. B, C, D, G, K. Kruskal-Wallis, Dunn's multiple comparisons.

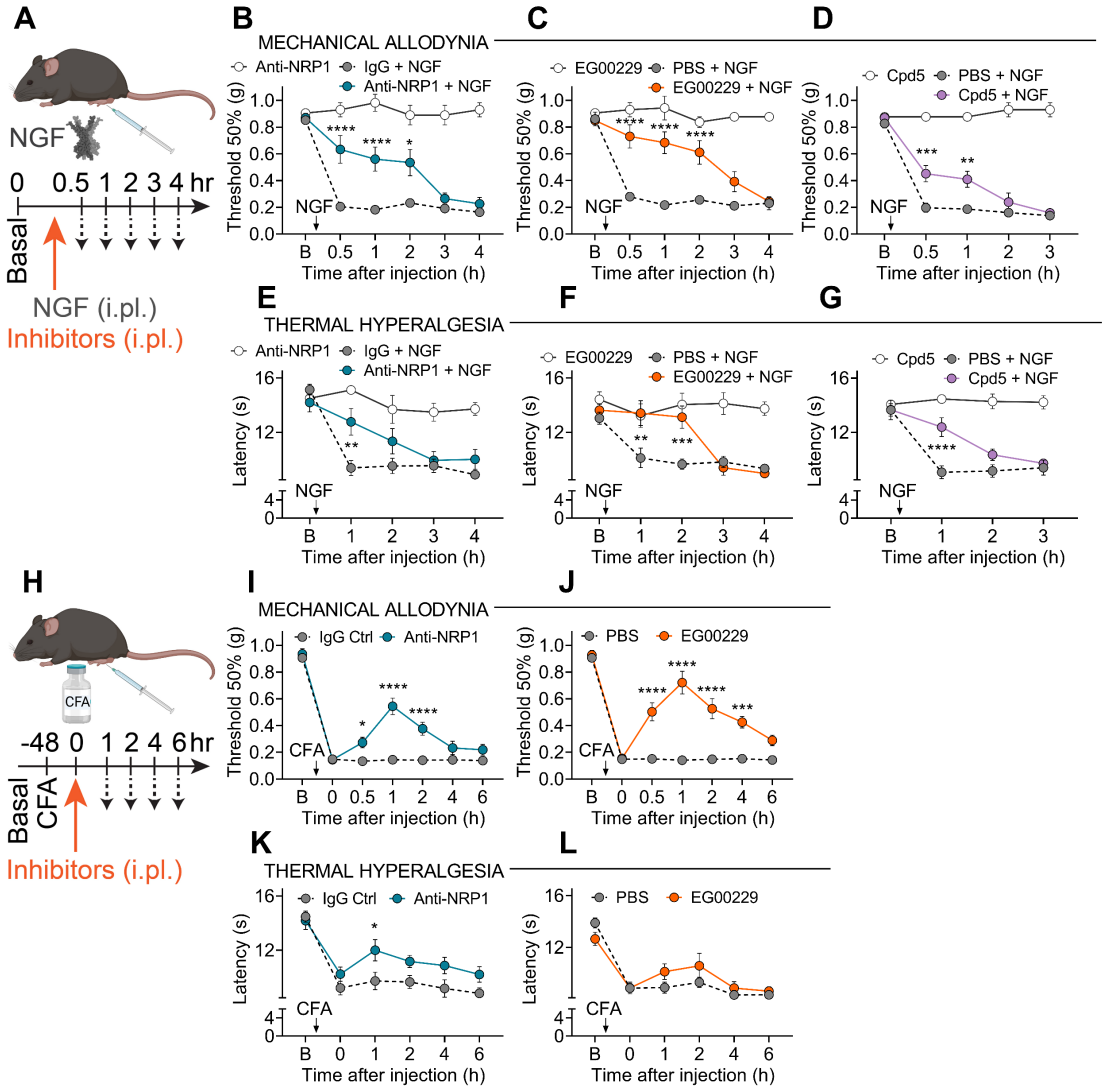


Figure 4. NRP1 inhibition abrogates NGF- and CFA-induced nociception in mice. (**A-G**) NGF-induced nociception. Effects of an antibody against the b1 domain of NRP1 (**B, E**; 7 µg/10 µl i.pl.), or NRP1 inhibitors EG00229 (**C, F**; 30 µM/10 µl i.pl.) or Compound 5 (**D, G**; Cpd5, C,F; 30 µM/10 µl i.pl.). After baseline ("B") measurements, inhibitors were co-injected with murine NGF (50 ng/10 µl, i.pl.). Mechanical allodynia (**B-D**) and thermal hyperalgesia (**E-G**) were measured. n=5-8 mice per group. **(H-L)** CFA-induced Inflammatory pain. Effects of anti-NRP1 (**I, K**) or EG00229 (**J, L**). Inhibitors were injected (i.pl.) 48 h after CFA (i.pl.). Mechanical allodynia (**I, J**) and thermal hyperalgesia (**K, L**) were measured. n=8-9 mice per group. Mean±SEM. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001 vs. PBS or IgG Ctrl. B-G, I-L. 2-way ANOVA, Sídák's multiple comparisons.

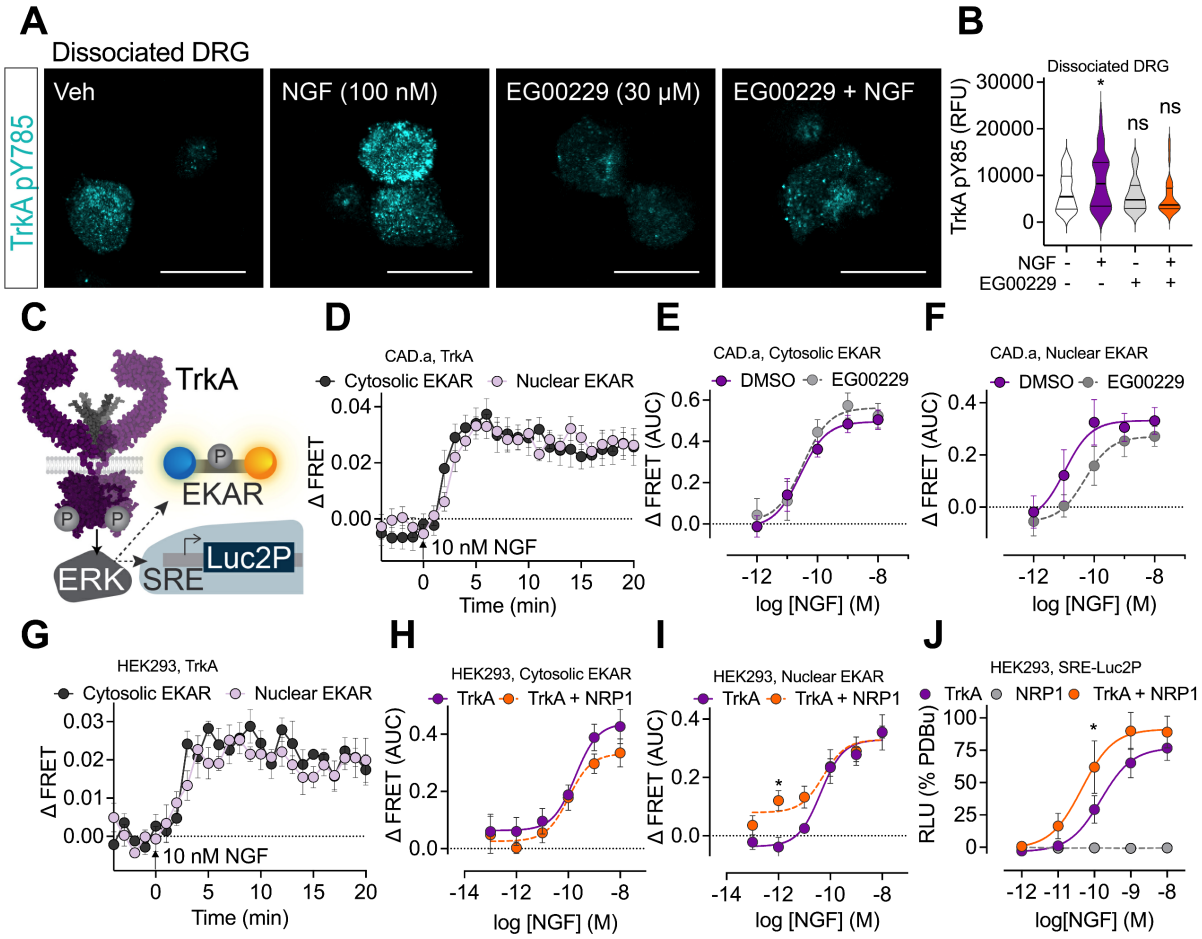
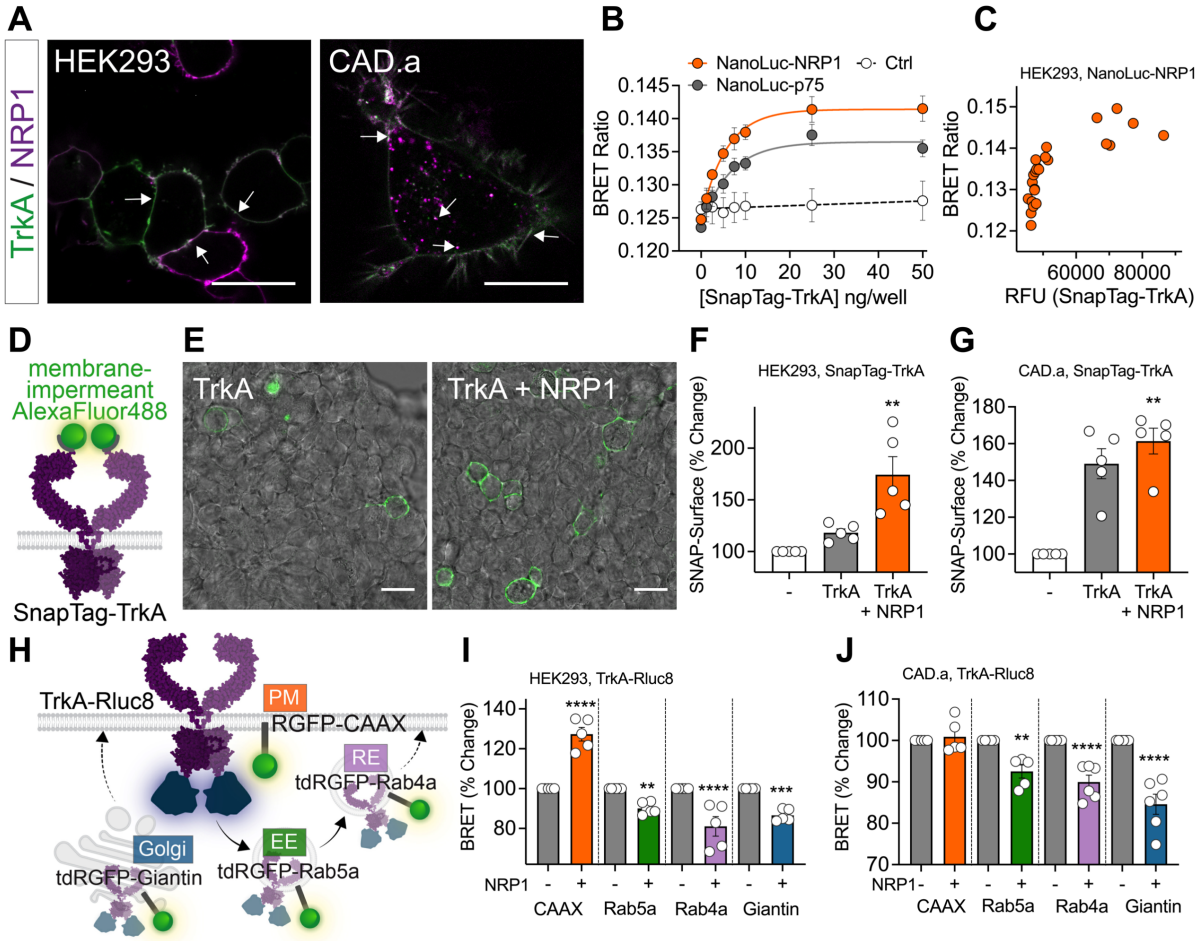


Figure 5. NRP1 modulates TrkA-mediated kinase signaling. (A-B) Effect of murine NGF (15 min) and NRP1 inhibitor EG00229 (30 min pre-incubation) on TrkA Y785 staining in mouse DRG neurons. Scale bar, 20 μm. RFU, relative fluorescence units. N=34-44 neurons from 3 independent experiments. (**C**) NGF-induced ERK signaling measured using FRET-based EKAR biosensors (**D-I**) or a downstream luciferase reporter (**J**). (**D-I**) NGF-induced modulation of ERK using biosensors localized to the cytosol (**D, E**) or nucleus (**D, F**) in neuron-like CAD.a cells expressing human TrkA. Kinetics of NGF-induced ERK monitored in CAD.a cells (**D**), comparing increasing NGF concentrations after pre-incubation with EG00229 (30 μM, 30 min) (**E-F**). (**G-I**) ERK signaling in HEK293T cells expressing TrkA alone or transfected with both TrkA and NRP1 (**H, I**). (**J**) Effect of increasing NGF concentrations on ERK transcription in cells expressing TrkA, NRP1 or both (% positive control, 10 μM PDBu). AUC, area under curve. RLU, relative luminescence units. Mean±SEM. Data from 4-8 independent experiments with triplicate wells. **P*<0.05, ****P*<0.001, *****P*<0.0001. B, I, J. 1-way ANOVA, Sídák's multiple comparisons.



686

687 **Figure 6. TrkA and NRP1 form a heteromeric complex**. (**A**) HEK293T or CAD.a cells expressing
688 SnapTag-TrkA and HaloTag-NRP1 simultaneously labeled with membrane-impermeant substrate
689 (SNAPTag-Alexa Fluor® 488, HaloTag-Alexa Fluor® 660). Representative images from n=5 independent
690 experiments. (**B-C**) BRET assays to monitor proximity between NanoLuc-NRP1 or NanoLuc-p75^{NTR} (10 ng)
691 and increasing SnapTag-TrkA DNA. Negative control, NanoLuc-TrkA and SnapTag-CALCRL.
692 Representative replicate (**C**) plotting BRET against relative fluorescence units (RFU) to quantify protein
693 expression. (**D-G**) Isolating cell-surface TrkA, imaged in HEK293T overlayed with phase-contrast imaging
694 in the absence or presence of NRP1 coexpression (**E**). (**F-G**) Quantification comparing fluorescence without
695 receptor (-), SnapTag-TrkA alone or SnapTag-TrkA co-transfected with NRP1 in HEK293T (**F**) or CAD.a
696 (**G**) cells. (**H-J**) BRET between TrkA tagged with *Renilla* luciferase (Rluc8) and *Renilla* green fluorescent
697 protein (RGFP) tagged markers of the plasma membrane (PM, RGFP-CAAX), early endosome (EE,
698 tdRGFP-Rab5a), recycling endosomes (RE, tdRGFP-Rab4a) or the cis-Golgi apparatus (tdRGFP-Giantin).
699 HEK293T cells (**I**) or CAD.a cells (**J**) were transfected with TrkA-Rluc8 in the absence (-) or presence (+)
700 of NRP1 co-expression. BRET was normalized relative to TrkA-Rluc8 alone (100%). Data from 5-6
701 independent experiments with triplicate wells. Mean±SEM. Scale bar, 20 µm. F, G. Paired t-test. I, J. 1-way
702 ANOVA with Šídák's multiple comparisons. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

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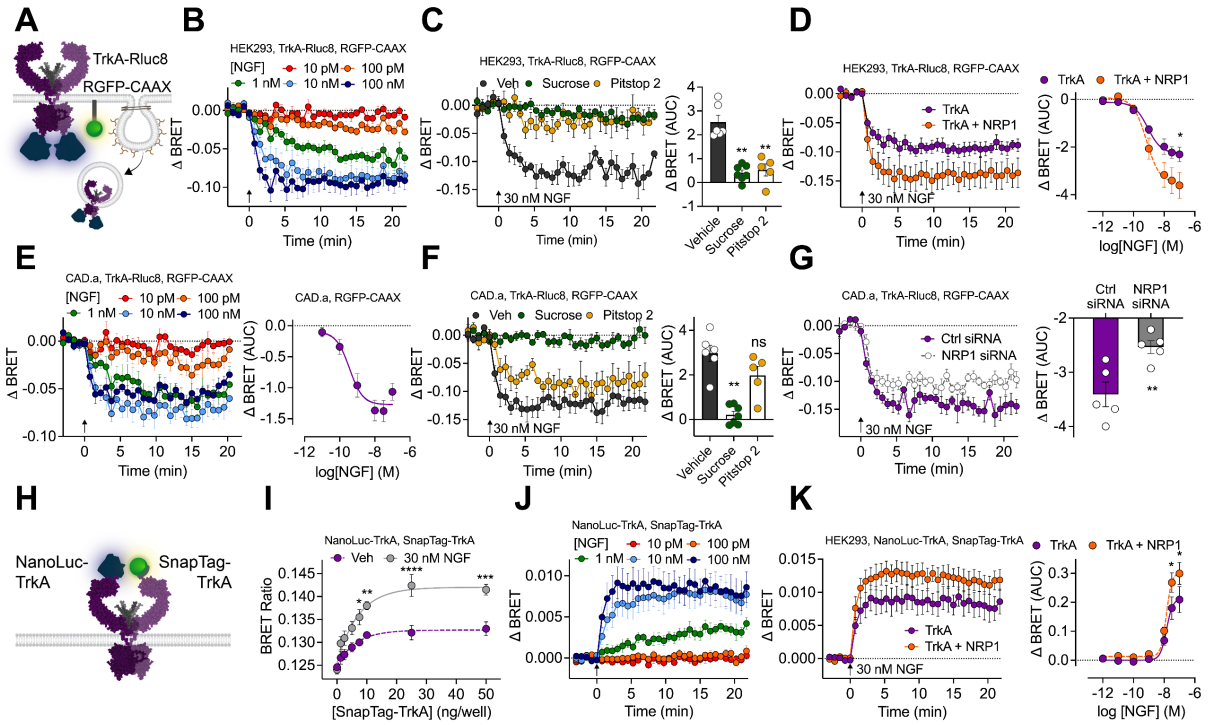

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705 **Figure 7. NRP1 modulates NGF-induced TrkA trafficking and RTK oligomerization**. (**A-G**) BRET was
706 measured at 37ºC between Rluc8-tagged TrkA and a fluorescent marker of the plasma membrane (RGFP-
707 CAAX). Decreased BRET indicates removal from the plasma membrane (**A**). (**B-D**) Trafficking in HEK293T
708 cells incubated with increasing NGF concentrations (**B**), or pre-incubated for 30 with hypertonic sucrose
709 (0.45 M), clathrin inhibitor pitstop 2 (30 μM) or vehicle (Veh) (**C**). Effect of NRP1 co-expression (**D**). (**E-G**)
710 Trafficking in CAD.a cells expressing human TrkA with increasing NGF concentrations (**E**), pre-incubation
711 with endocytic inhibitors (**F**) with transfected with control or murine NRP1 siRNA (**G**). (**H-K**) BRET measured
712 between NanoLuc- and SnapTag- TrkA to measure RTK oligomerization in HEK293T cells. (**I**) BRET with
713 increasing SnapTag-TrkA and fixed NanoLuc-TrkA (10 ng), after 30 min incubation with vehicle or NGF. (**J-**
714 **K**) Using a fixed donor:acceptor ratio (1:2.5), oligomerization kinetics in response to increasing NGF
715 concentrations (**J**) in the absence and presence of NRP1 co-expression (**K**). Kinetic measurements were
716 made at 37ºC. Data from 4-6 independent experiments with triplicate wells. AUC, area under curve.
717 Mean±SEM. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001. G, Unpaired t-test. D, I, K. 1-way ANOVA with
718 Sídák's multiple comparisons.

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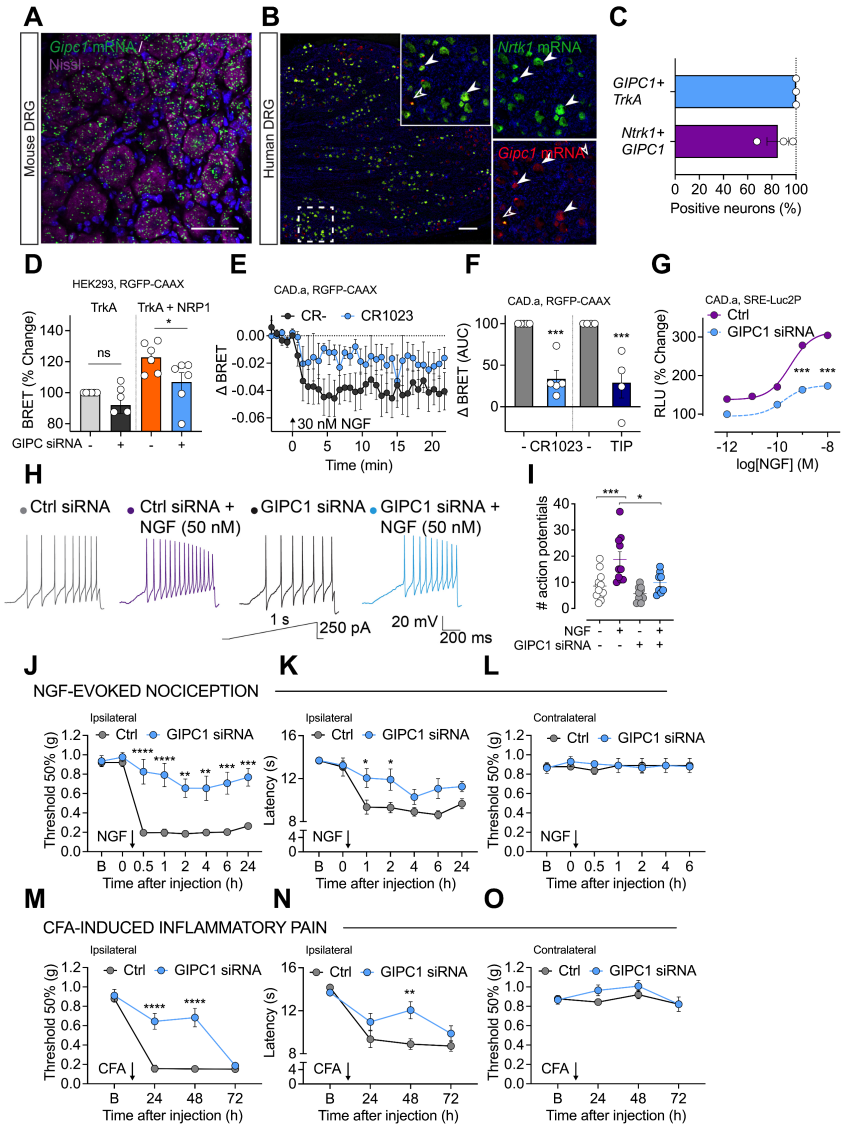

Figure 8. GIPC1 modulates TrkA trafficking, signaling and NGF-induced nociception. (**A-C**) RNAScope® localization of *Gipc1* mRNA in mouse DRG (**A**) or *Ntrk1* and *Gipc1* mRNA in human DRG (**B, C**). Arrows indicate mRNA expression within the same cell. Representative images, n=5 mice; n=3 human sections. Scale bar, 500 µm. (**D**) Effect of GIPC1 siRNA on increased TrkA at the plasma membrane under basal conditions upon NRP1 co-expression in HEK293T cells measured using BRET. (**E-F**) Effect of 30 min pre-incubation of GIPC1 antagonist (300 µM CR1023 or negative control) or myosin VI inhibitor (50 µM 2,4,6-Triiodophenol, TIP) on NGF-induced TrkA-Rluc8 trafficking from a marker of the plasma membrane (RGFP-CAAX) in CAD.a cells. (**G**) Effect of GIPC1 siRNA on NGF-induced downstream ERK transcription in CAD.a cells. RLU, relative luminescence units. Data from 5-6 independent experiments with triplicate wells. (**H-I**) Sample traces of action potential firing in mouse DRG neurons evoked by injecting a 1 s ramp pulse from 0 to 250 pA (**G**), with the number of evoked action potentials (**H**). N=7-10 cells. (**J-O**) Effects of GIPC1 siRNA (i.t.) on NGF (50 ng/10 µl, i.pl.)-induced (**J-L**) or CFA-induced (**M-O**) mechanical allodynia (**J-K,M-N**) and thermal hyperalgesia (**L,O**) in mice, including mechanical allodynia tested in the contralateral hind paw after NGF (**K**) or CFA injection (**N**). N=6-8 mice per group. AUC, area under curve. 'B', basal. Mean±SEM. C,E. 1-way ANOVA, Sídák's multiple comparisons. H, Tukey's multiple comparison. I-O, 2-way ANOVA, Sídák's multiple comparisons. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

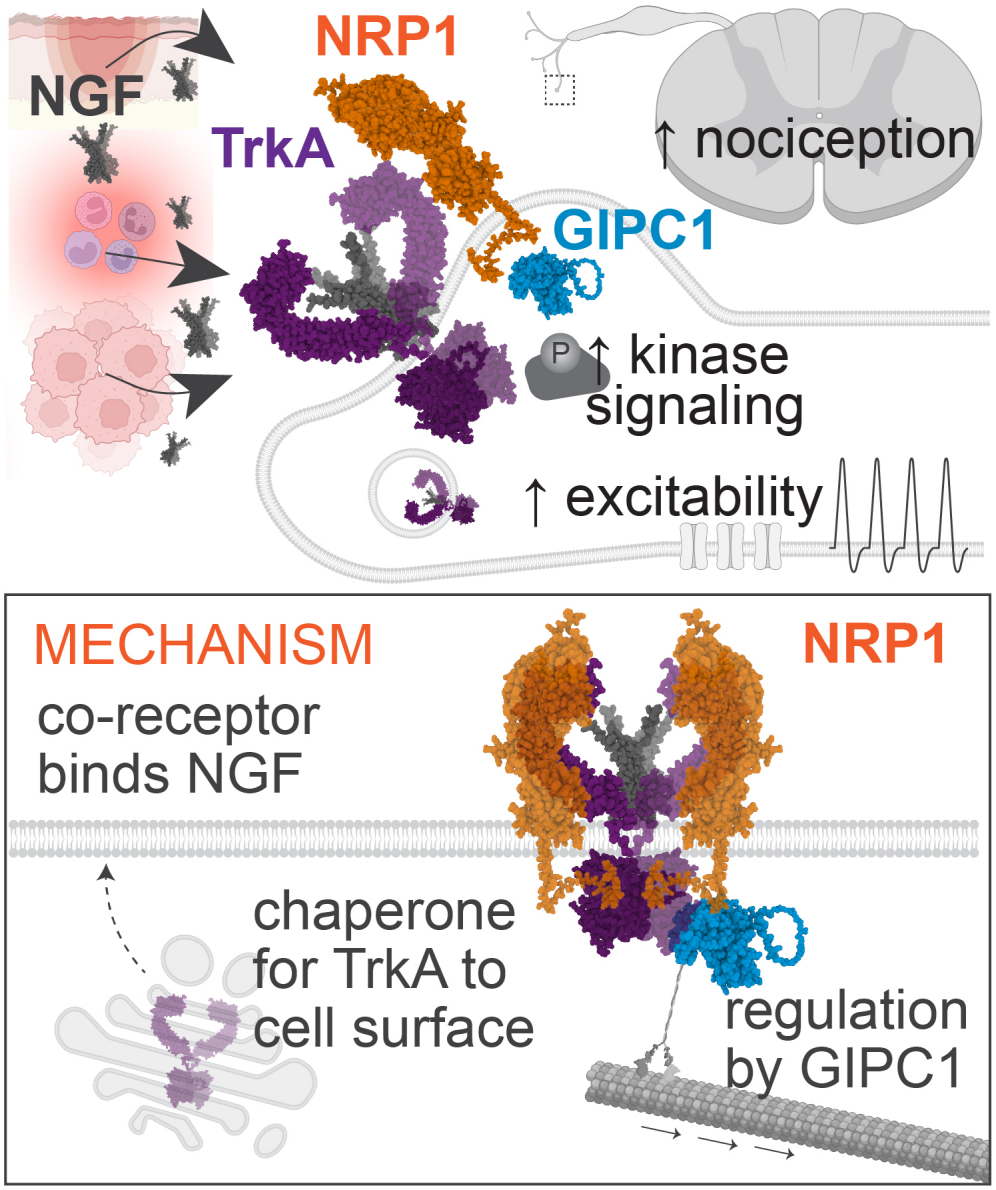

Figure 9. Proposed mechanism for NRP1- and GIPC1-mediated NGF/TrkA signaling. NGF released from sites of injury, inflammation or the tumor microenvironment act on TrkA expressed by primary afferent neurons. NRP1 and GIPC1 potentiate kinase signaling, neuronal excitability and nociception. As a co-receptor, NRP1 can directly bind NGF and act as a chaperone, mediated by interactions with adaptor protein GIPC1.

743 **TABLES**

744 **Suppl. Table 1. Gating properties of Ca^{2+} and Na^{+} currents recorded from mouse DRG neurons.**

Total Ca^{2+}	**DMSO (0.1%)**	**EG00229 (30 µM)**	**NGF (50 nM)**	**EG00229 + NGF**
Activation				
$V_{1/2}$	5.793 ± 1.891 (10)	-2.828 ± 0.871 (6)†	5.834 ± 1.995 (9)††	-0.473 ± 1.265 (8)
k	7.756 ± 1.534 (10)	4.230 ± 0.731 (6)	7.972 ± 1.608 (9)	6.428 ± 1.109 (8)
Inactivation				
$V_{1/2}$	-27.372 ± 1.919 (9)	-20.137 ± 0.901 (7)	-23.909 ± 3.080 (9)	-20.166 ± 1.314 (8)
k	-14.944 ± 2.109 (9)	-10.623 ± 0.842 (7)	-15.079 ± 3.305 (9)	-13.176 ± 1.287 (8)
Total Na^{+}	**DMSO (0.1%)**	**EG00229 (30 µM)**	**NGF (50 nM)**	**EG00229 + NGF**
Activation				
$V_{1/2}$	-22.436 ± 1.150 (10)	-23.229 ± 0.499 (13)	-23.151 ± 0.418 (14)	-23.600 ± 0.993 (11)
k	5.098 ± 1.014 (10)	3.580 ± 0.434 (13)	3.432 ± 0.362 (14)	5.145 ± 0.877 (11)
Inactivation				
$V_{1/2}$	-48.736 ± 4.219 (10)	-43.306 ± 4.249 (13)	-54.450 ± 2.244 (14)	-38.622 ± 4.013 (11)*
k	-18.568 ± 4.934 (10)	-21.036 ± 5.439 (13)	-14.748 ± 2.243 (14)	-19.732 ± 4.708 (11)

745 Values are mean±SEM calculated from fits of the data from the indicated number of individual cells (in
746 parentheses) to the Boltzmann equation; $V_{1/2}$ midpoint potential (mV) for voltage-dependent of activation or
747 inactivation; *k*, slope factor. Data were analyzed with 1-way ANOVA with Tukey's multiple comparisons. †
748 p=0.0115 for $V_{1/2}$ activation of EG00229 vs DMSO (0.1%); †† p=0.0131 for $V_{1/2}$ activation of NGF vs
749 EG00229; * p= 0.0175 for $V_{1/2}$ inactivation of EG00229 + NGF vs DMSO (0.1%).

750 **Suppl. Table 2. Pharmacological parameters (pEC_{50}) of NGF derived in at human TrkA, in the**
751 **absence or presence of NRP1 modulation**.

CAD.a cells	**Control**	**NRP1 inhibition (EG00229)**
Cytosolic ERK (FRET)	10.64 ± 0.41 (5)	10.53 ± 0.17 (5)
Nuclear ERK (FRET)	11.09 ± 0.33 (5)	10.01 ± 0.38 (5) *
HEK293T cells	**Control**	**NRP1 overexpression (HaloTag)**
Cytosolic ERK (FRET)	9.89 ± 0.21 (5)	9.87 ± 0.10 (4)
Nuclear ERK (FRET)	10.37 ± 0.19 (5)	10.59 ± 0.33 (5)
ERK Transcription (SRE-Luc2P)	9.67 ± 0.48 (8)	10.15 ± 0.57 (8) ***
TrkA-Rluc8 Trafficking (BRET)	9.06 ± 0.10 (6)	9.08 ± 0.13 (6)
TrkA/TrkA Oligomerization (BRET)	7.81 ± 0.07 (4)	7.87 ± 0.04 (5)

752 Derived in HEK293T or neuron-like CAD.a cells transfected with full-length, human TrkA. Values are
753 mean±SEM calculated from non-linear regressions fit of the data from independent replicates (in
754 parentheses), each with triplicate wells. Quantified as the negative antilog of the half-maximal effective
755 concentration (pEC_{50} = -logEC_{50}). * p<0.05, *** p<0.001. Paired t-test.

756 **Suppl. Table 3. Summary of siRNA sequences used in this study**.

Target	Sequence
Control	UGGUUUACAUGUCGACUAA UGGUUUACAUGUUGUGUGA UGGUUUACAUGUUUUCUGA UGGUUUACAUGUUUUCCUA
mNrp1	GAAUUGCUGUGGAUGAUAU AGUAAGAGGUGUCAUCAUU CCACAAGGUUCAUCAGGAU GGAAUGUUCUGUCGCUAUG
hGipc1	GCACUCGGGCUCACCAUCA GGCCGUACCUUCACGCUGA GCAAGGCCUUCGACAUGAU CUGGAGAGUUACAUGGGUA
mGipc1	GCAUCGAGGGCUUCACUAA CGUCGGCCUUUGAGGAGAA GUGGAUGACUUGCUAGAGA GCUGAGGCCUUCCGACUAC

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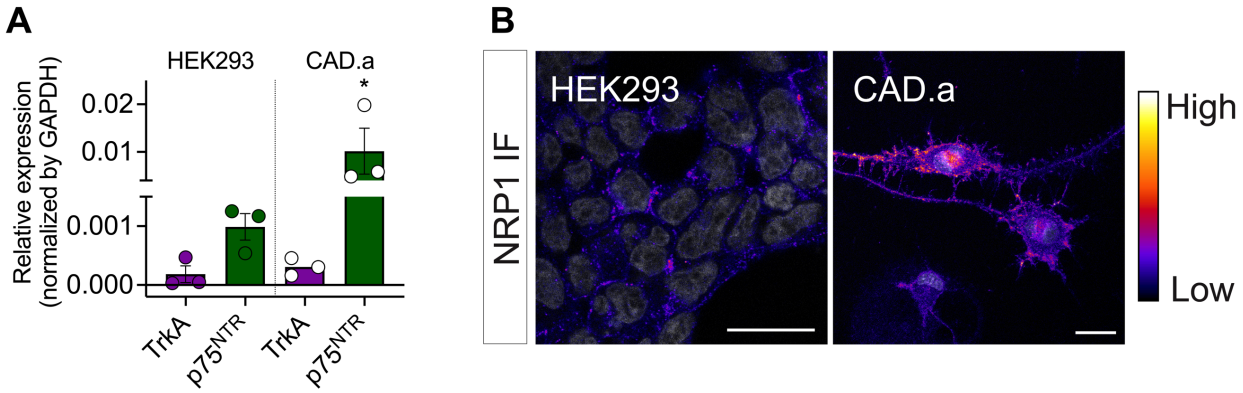
758 **SUPPLEMENTARY FIGURES**



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760 **Supplementary Figure 1. Characterization of receptor expression in neuron-like CAD.a cells**. (**A**)
761 Quantification of expression of *Ntrk1* mRNA (TrkA) or *Ngfr* ($p75^{NTR}$) in HEK293T or CAD.a cells determined
762 by quantitative RT-PCR. Data from 3 independent experiments with triplicate wells. (**B**) Immunofluorescent
763 (IF) staining of NRP1 in HEK293T or CAD.a cells using vesencumab. Scale bar, 20 µm. Representative
764 from 4 independent experiments. Mean±SEM. A, 1-way ANOVA, Dunnett's multiple comparisons. * $P<0.05$.

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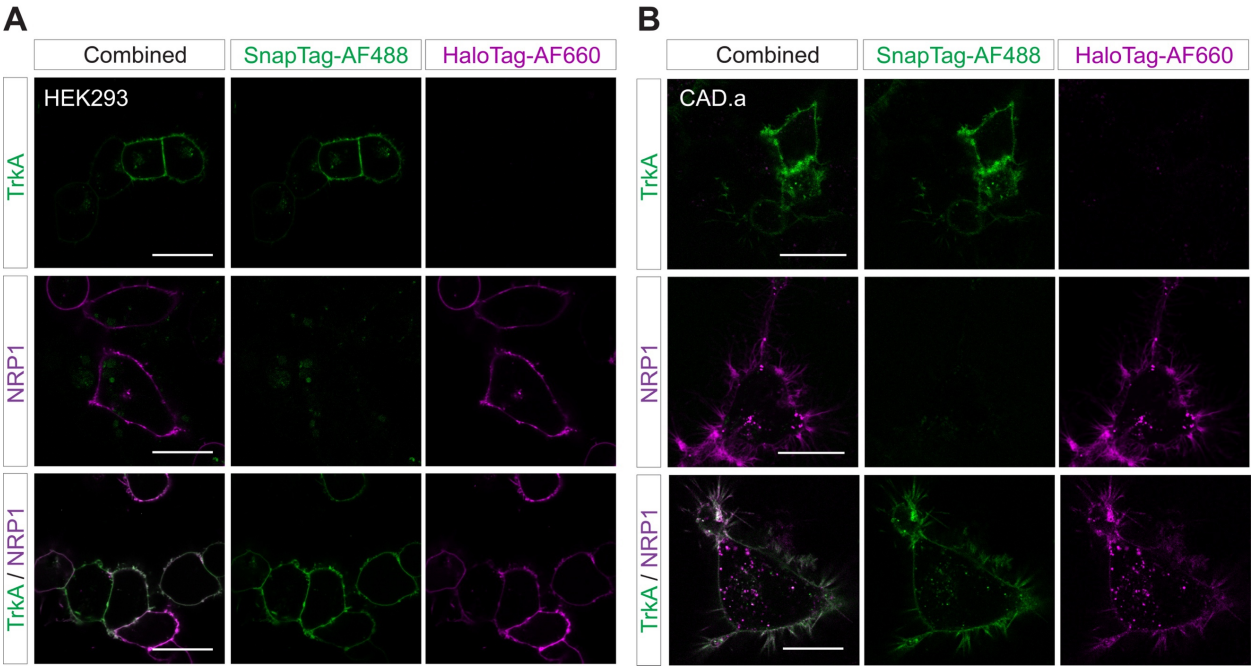

Supplementary Figure 2. Specificity of SnapTag- and HaloTag- labeling in live cells. HEK293T cells (***A***) or CAD.a cells (***B***) were transfected with SnapTag-TrkA, HaloTag-NRP1 or both (1:2 TrkA to NRP1 ratio). Tags were simultaneously labeled with membrane-impermeant substrate (SNAPTag-Alexa Fluor® 488, HaloTag-Alexa Fluor® 660). Representative from n=5 independent experiments. Scale bar, 20 µm.

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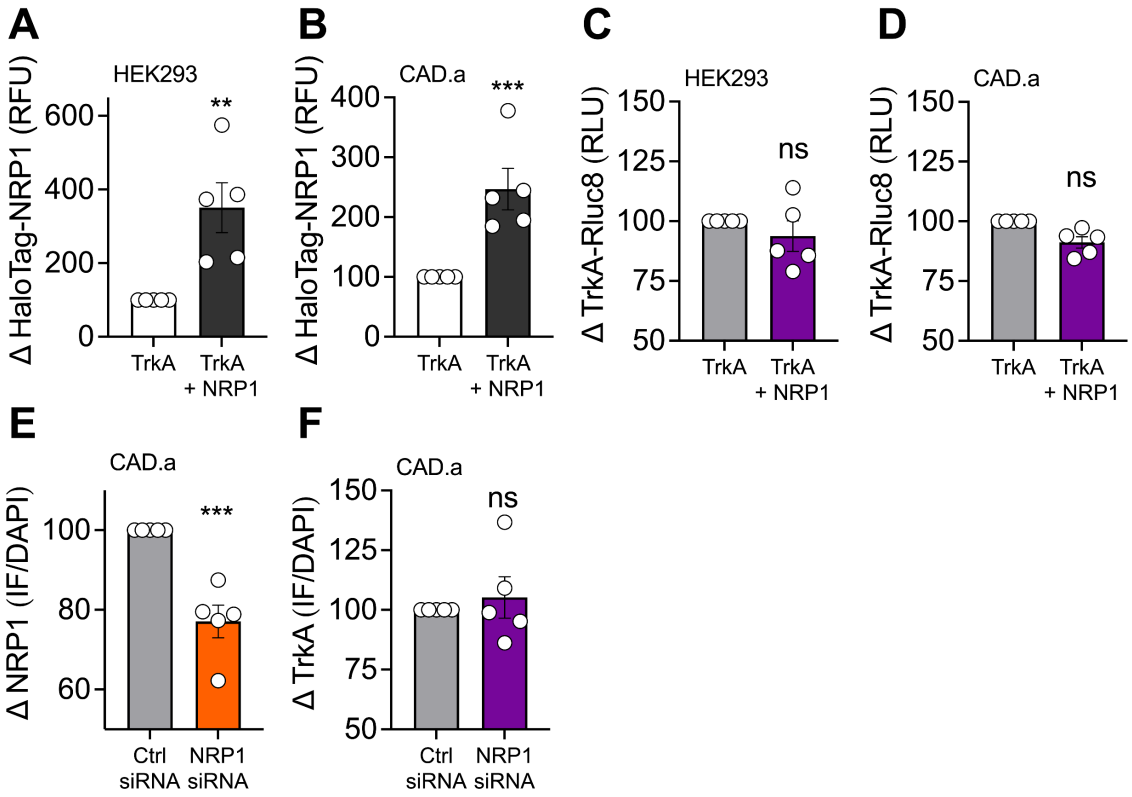
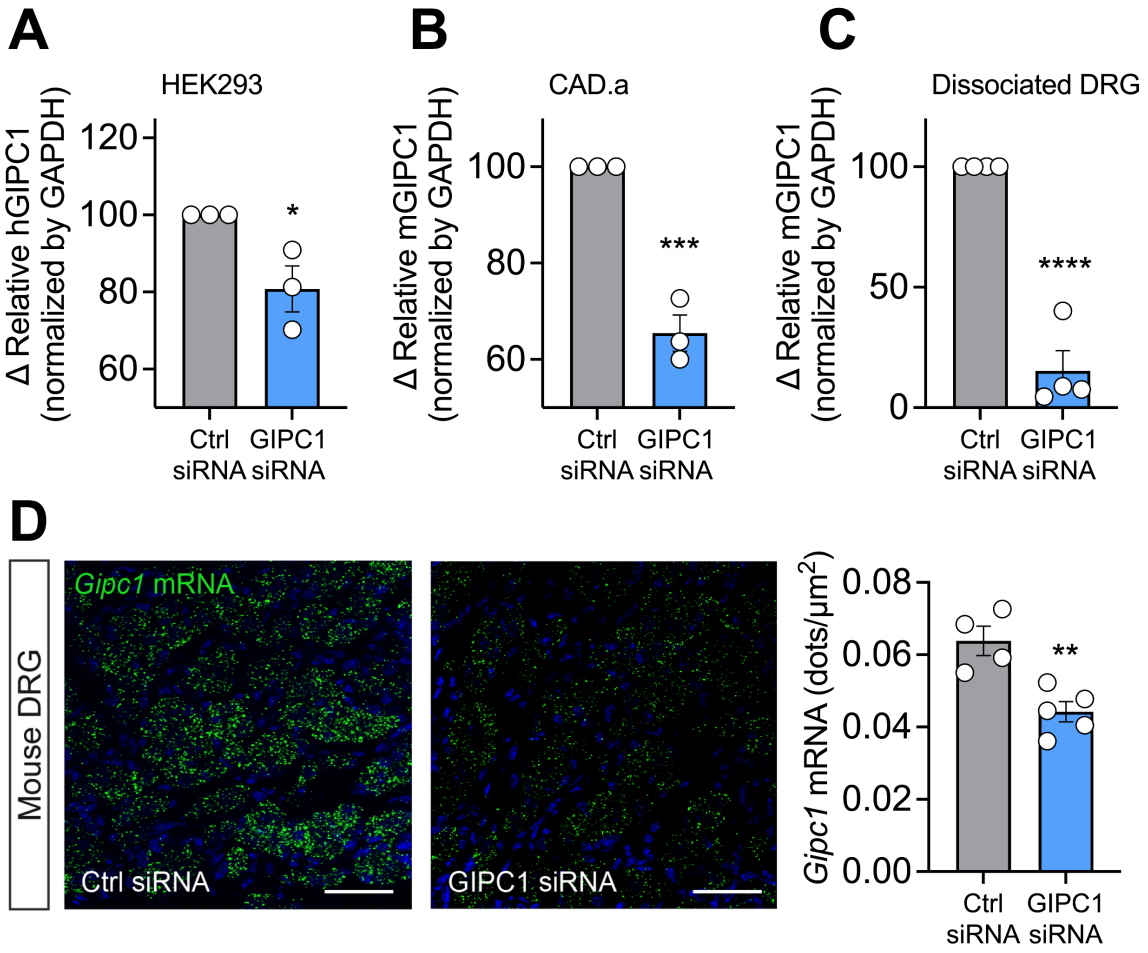


772

773 **Supplementary Figure 3. Effect of co-expression or siRNA on TrkA and NRP1 expression**. (**A-D**)
774 HEK293T (***A,C***) or CAD.a (***B,D***) cells expressed TrkA-Rluc8 in the absence or presence of HaloTag-NRP1.
775 Cells were labeled with membrane-impermeant HaloTag-Alexa Fluor® 660 and emissions were measured
776 for AlexaFluor660 as relative fluorescence units (RFU) (***A,B***). Alternatively, TrkA-Rluc8 emissions were
777 measured after incubation with coelenterazine purple to infer TrkA expression as relative luminescence
778 units (RLU) (***C,D***). (**E-F**) Effect of NRP1 siRNA on expression of NRP1 (***E***) or TrkA (***F***) quantified using IF
779 staining normalized to nuclei count (DAPI). Data expressed as percentage change over control (100%).
780 Mean±SEM. A-D, G, H. Unpaired t-test. ***P<0.01, *** P<0.001.



Supplementary Figure 4. Confirmation of GIPC1 knockdown using siRNA. (**A-C**) Quantification of expression of *Gipc1* mRNA in HEK293T (***A***), CAD.a cells (***B***) or cultured dissociated DRG neurons (***C***) in the presence of control (Ctrl) or GIPC1 siRNA determined by quantitative RT-PCR. Data from 3-4 independent experiments with triplicate wells. (**D**) RNAScope® localization of *Gipc1* mRNA in murine DRG after intrathecal injection of control or GIPC1 siRNA. Representative from n=5 mice. A-D. Unpaired t-test. *P<0.05, **P<0.01, *** P<0.001, **** P<0.0001.

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