Etv4 regulates nociception by controlling peptidergic sensory neuron development and peripheral tissue innervation

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Abstract

The perception of noxious environmental stimuli by nociceptive sensory neurons is an essential mechanism for the prevention of tissue damage. Etv4 is a transcriptional factor expressed in most nociceptors in dorsal root ganglia (DRG) during the embryonic development. However, its physiological role remains unclear. Here, we show that Etv4 ablation results in defects in the development of the peripheral peptidergic projections in vivo and deficits in axonal elongation and growth cone morphology in cultured sensory neurons in response to NGF. From a mechanistic point of view, our findings reveal that NGF regulates Etv4-dependent gene expression of molecules involved in extracellular matrix (ECM) remodeling. *Etv4*-null mice were less sensitive to noxious heat stimuli and chemical pain and this behavioral phenotype correlates with a significant reduction in the expression of the pain-transducing ion channel TRPV1 in mutant mice. Together, our data demonstrate that Etv4 is required for the correct innervation and function of peptidergic sensory neurons, regulating a transcriptional program that involves molecules associated to axonal growth and pain transduction.

Introduction

Understanding the development and establishment of neural circuits that regulate pain transduction is an important issue in neural development. However, little is known about the molecular mechanisms underlying this process. Primary afferent neurons, located in the dorsal root ganglion (DRG), are sensory neurons with the capacity to detect and transduce external physical stimuli into electrical signals that are then conveyed to the central nervous system (CNS). Primary afferent neurons have a single axon that bifurcates into a peripheral branch, that innervates diverse targets such as the skin or muscle, and a central branch, that establishes synaptic contact with CNS neurons. According to their perception modalities, sensory neurons can be classified into proprioceptors, which sense body positions, mechanoreceptors, which mediate touch, and nociceptors, which are involved in the perception of pain and temperature (Basbaum et al., 2009). These major subpopulations exhibit different physiological, morphological and molecular properties (Usoskin et al., 2015).

It has been established that the expression of neurotrophic factor receptors defines the functionality of DRG neurons (Marmigere and Ernfors, 2007). Neurons that express TrkA respond to nerve growth factor (NGF); while $TrkB^+$ or $TrkC^+$ are activated by brain-derived neurotrophic factor (BDNF) and neurotrophin 4/5 (NT4/5) or neurotrophin 3 (NT3), respectively. These neurothrophic signals are essential for the peripheral innervation of appropriate targets, cell survival and the expression of ion channels, receptors and molecules defining their functional properties (Lallemend and Ernfors, 2012; Meltzer et al., 2021).

Neurons that express the TrkA receptor during embryonic development project fibers into the epidermis in response to NGF and originate nociceptive neurons. Animals deficient in TrkA and NGF lack nociceptive neurons and their projections, and are defective in response to noxious stimuli (Carroll et al., 1992; Crowley et al., 1994; Smeyne et al., 1994). In humans, mutations in TrkA or NGF have been associated to deficits in nociceptor development and pain sensation (Einarsdottir et al., 2004; Indo, 2010; Indo et al., 1996). Later throughout development, these neurons segregate into two subpopulations, peptidergic and non-peptidergic neurons. Peptidergic nociceptors are defined by the expression of calcitonin gene-related peptide (CGRP) and other specific neuropeptides, such as substance P (Ju et al., 1987; Xu et al., 1990) and maintain the expression of TrkA. On the other hand, non-peptidergic neurons extinguish the expression of TrkA and respond to Glial-Derived Neurotrophic Factor (GDNF) family ligands (GFLs) through the receptor tyrosine kinase, Ret, and its coreceptor molecules GFRαs (Lallemend and Ernfors, 2012; Meltzer et al., 2021).

As mentioned, in adult DRGs, TrkA expression exclusively persists in peptidergic neurons, and signaling through NGF plays an important role in setting pain sensitivity. Many reports indicate that endogenous NGF levels are elevated in chronic pain (Denk et al., 2017; Pezet and McMahon, 2006; Woolf et al., 1994). Moreover, the administration of NGF in the periphery leads to hyperalgesia (Dyck et al., 1997; Lewin et al., 1993; Petty et al., 1994; Rukwied et al., 2010).

Different ion channels expressed in peptidergic neurons are involved in the transduction of painful stimuli, including the Transient Receptor Potential Vanilloid 1 (TRPV1), which is a heat- and capsaicin-evoked channel expressed predominantly by peptidergic afferents (Caterina et al., 1997; Yang and Zheng, 2017). The genetic deletion of the *Trpv1* gene

results in an impaired response to heat stimuli and capsaicin (Caterina et al., 2000). Interestingly, the expression of TRPV1 and its phosphorylation levels are controlled by the activity of NGF/TrkA receptor (Amaya et al., 2004; Ji et al., 2002; Xue et al., 2007; Zhang et al., 2012; Zhu and Oxford, 2007).

NGF signaling in sensory neurons involves its binding to the TrkA receptor at the axon terminal, subsequent activation of the MEK/ERK and PI3K/Akt pathways, and retrograde signal transduction to the nucleus where it triggers a specific transcriptional program. This signaling is essential for neuronal viability, neurite outgrowth and pain sensitivity transduction (Chowdary et al., 2012; Harrington and Ginty, 2013). In mammals, members of the Pea3 (polyoma enhancer activator 3) subfamily of ETS (E26 transformation specific) transcription factors have been implicated as mediators of neurotrophic factor signaling downstream of the MEK/ERK pathway and in regulating the establishment of synaptic connections (Arber et al., 2000; Fontanet et al., 2013). This family includes three members: Etv1, Etv4 and Etv5, also named Er81, Pea3 and Erm, respectively. These factors have been associated with the development and progression of different types of tumors and also with tissue remodeling processes (de Launoit et al., 2006; Oh et al., 2012; Oikawa and Yamada, 2003; Yordy and Muise-Helmericks, 2000). In particular, Etv4 was found to be highly expressed in embryonic DRG $TrkA^+$ sensory neurons. Furthermore, in vitro studies have shown that Etv4 is induced by distal NGF and is required for proper axonal growth of $TrkA^+$ neurons (Fontanet et al., 2013). However, the physiological role of Etv4 in the development of the peripheral nervous system (PNS) remains unclear.

We show herein that Etv4 is required for a correct cutaneous peptidergic innervation and pain transduction. Our findings indicate that Etv4 is necessary for the expression of enzymes involved in extracellular matrix (ECM) remodeling, and for the proper formation of growth cones that mediate NGF-dependent axonal growth. We also demonstrate that Etv4 mutant mice are less susceptible to heat nociception and chemical stimuli. Furthermore, we provide evidence indicating that Etv4 is essential for the development of nociceptors, by mediating the expression of the pain transducing channel, TRPV1.

Results

Etv4 is required for proper epidermal innervation of peptidergic sensory neurons

Based on the fact that Etv4 mediates NGF signaling (Fontanet et al., 2013), and that NGF is required for the epidermal innervation of DRG neurons that express TrkA (peptidergic nociceptive cells) during embryonic development (Patel et al., 2000), we examined whether Etv4 deletion plays a role in such event by analyzing *Etv4*-deficient mice. For this purpose, we examined presence of free nerve endings in transverse sections of the glabrous skin of the hindpaw of *Etv4*$^{-/-}$ and wild-type (wt) mice at two different developmental stages (newborn P0 and 2-month-old mice). The peptidergic fibers were visualized using antibodies against calcitonin gene-related peptide (CGRP), which label neurons that respond to NGF. In these assays, we found fewer CGRP^{+} axon terminals penetrating through the basal membrane into the epidermis in *Etv4*-null mice compared to wild-type littermates at P0 (Fig. 1A and C). A similar deficiency in CGRP^{+} epidermal innervation was also observed in adult *Etv4*$^{-/-}$ compared to wild-type (Fig. 1B and D). Likewise, epidermal CGRP^{+} fibers were shorter in adult mutant than in control mice (Fig. 1E). This shortening did not result from external constraints such as a reduced thickness of the skin, as no difference in skin thickness was observed between control and *Etv4*-deficient mice (data not shown). These findings indicate that developing peptidergic nociceptive sensory neurons do not adequately innervate the epidermis in the absence of Etv4. In order to analyze epidermal innervation of non-peptidergic nociceptive neurons, we performed stainings using antibodies against the GFRα1/GFRα2 receptors, which labeled non-peptidergic DRG sensory fibers (De Vincenti et al., 2021; Sakai et al., 2017). Quantification of this innervation indicated that the density of cutaneous non-pepetidergic fibers was unchanged between wild-type and *Etv4-KO* adult mice (Fig 1F and G). Taken together, these observations indicate that Etv4 is essential for cutaneous peripheral peptidergic projections, but is not required for non-peptidergic innervation.

We next examined the expression of *Etv4* mRNA in DRG at different embryonic and postnatal stages, using RT-PCR. Interestingly, while *Etv4* mRNA is expressed during embryonic development, its expression decreases in the postnatal ages (Fig.1H). Immunostaining of DRG sections of E14.5 and P20 mice confirmed this result at the protein level, indicating that at embryonic stages Etv4 colocalizes with a great number of TrkA-positive neurons and that after birth its levels are substantially downregulated

(Fig.1I). The specificity of the Etv4 antibodies was evaluated on tissue from *Etv4*-deficient mice (Fig S1) (Fontanet et al., 2018). In agreement with previous work, we observed that the vast majority of TrkA-positive neurons express Etv4 at early developmental stages (Fontanet et al., 2013). However, the expression of Etv4 exceeds the TrkA-positive cell population, opening the possibility that Etv4 might have physiological contribution in other population of DRG neurons. At postnatal stages we observed the presence of Etv4 in the nuclei of TrkA-positive but also in TrkA-negative neurons, indicating that Etv4 expression is not exclusive of peptidergic cells (Fig 1I).

To assess whether Etv4 is required for the proper development of central spinal projections of CGRP$^+$ afferents, we evaluated the distribution of CGRP projections in tissue from *Etv4*-deficient and wild-type animals at P15. In wild-type mice, CGRP-labeled fibers were found extending into the dorsal horn and projecting predominantly towards the superficial lamina I and II. No differences were observed either in the area covered by CGRP$^+$ fibers nor in the number of CGRP$^+$ terminals that innervate the dorsal horn of the spinal cord between *Etv4* mutant and wild-type mice (Fig. 2). Moreover, aberrant CGRP$^+$ projections were not detected in the spinal cord of *Etv4*-deficient mutant mice compared to wild-type animals (Fig. 2D). Intriguingly, a reduction in the intensity of CGRP$^+$ staining was detected in *Etv4* null mice (Fig. 2E). Despite this, no differences in CGRP mRNA levels were observed between wild-type and *Etv4-KO* mice (data not shown). To further analyze the role of Etv4 in peptidergic central innervation, we labeled central spinal projections of small-diameter neurons by TrkA staining. No differences were observed neither in the area covered by TrkA-positive fibers nor in the intensity of the TrkA-positive terminals innervating the spinal cord dorsal horn between *Etv4*-mutant and wild-type mice (Fig. 2 C and F). Thus, together these findings suggest that Etv4 affects the morphology and density of peripheral but not central axons of peptidergic neurons.

Etv4 is dispensable for DRG neuronal viability

Based on the defects in peptidergic epidermal innervation observed in *Etv4*-null mice, we decided to further analyze whether the Etv4 deletion affects neuronal viability in the development of DRG sensory neurons. To this end, sections of P15 *Etv4*-mutant and wild-type mice were stained with the pan-neuronal marker NeuN or with the sensory

neuron marker PGP9.5. No significant differences were observed in the total number of sensory neurons, suggesting that Etv4 is not associated with the survival of DRG neurons (Fig. 3A and B), and therefore the defect observed in peripheral peptidergic innervation is not a consequence of a change in neuronal viability. According to these data, the size of lumbar sensory ganglia of the Etv4 mutants was comparable to that of wild-type mice (Fig. S2).

Then, we decided to examine whether the altered epidermal innervation in *Etv4*-knockout (KO) mice could be explained by changes in the number of nociceptive neurons. As mentioned above, these neurons are classified into non-peptidergic, expressing the GFL receptor Ret and GFRα1/2, or peptidergic, characterized by the expression of TrkA. No changes were observed in the percent of $TrkA^+$ cells relative to the total number of DRG neurons ($NeuN^+$) in *Etv4*- mutant mice compared to wild-type mice in young P15 animals (Fig. 3A and C). The expression of *TrkA*, *Ret* and *GFRα1* mRNAs was analyzed by RT-PCR. No differences were observed in the expression levels of the mRNA of these receptors in *Etv4*-deficient mice compared to wild-type animals (Figure 3D-E).

Taken together, these results allow us to conclude that whereas Etv4 ablation does not impact neither the number of $TrkA^+$ nociceptive neurons, nor the expression of neurotrophic factor receptors, it leads to a deficit in peptidergic peripheral innervation. The change in peripheral innervation observed in *Etv4* mutants is therefore attributable neither to cell death, nor to a cell fate conversion.

Etv4 is required for axonal growth of NGF-dependent sensory neurons

To further characterize the role of Etv4 in axonal extension, we performed in vitro assays on dissociated DRG primary neurons. Embryonic DRG neurons from $Etv4^{-/-}$ or wild-type mice were cultured in the presence of NGF and the extent of axonal processes was measured after 24-36h. *Etv4* mutant neurons showed shorter neurites compared to neurons derived from wild-type animals (Fig. 4A-C). These differences were not due to variations in cell viability (Fig. 4D). In addition, the growth of cranial sensory neurons obtained from the trigeminal ganglia (TG), which also express Etv4 and respond to NGF, was analyzed (Fig. S3). Consistent with the results obtained using DRG neurons, the absence of Etv4 in TG sensory neurons also resulted in poor axonal growth in presence

of NGF (Fig. 4E-G). Therefore, our data indicate that Etv4 is required for proper axonal growth of the NGF-responsive sensory neurons.

Developing axons are guided to their target tissues by extracellular physical and molecular cues detected by the growth cones at their tips. Extracellular signals are locally integrated in the growth cone and converge on dynamic changes in the actin cytoskeleton that play a key role in generating the force required for neurite extension. Since Etv4 is involved in the growth of neurites, we decided to examine whether it does so by regulating the morphology of the growth cone. We measured growth cone area of *Etv4*-deficient and wild-type DRG and TG neurons, cultured in the presence of NGF. Cells were labeled with phalloidin, which stains polymerized actin. DRG sensory neurons derived from *Etv4*$^{-/-}$ mice exhibited a significant reduction in growth cone area compared to their wild-type counterparts, in the presence of NGF (Fig. 4H and I). A similar reduction in the growth cone size was observed in *Etv4*-deficient trigeminal sensory neurons (Fig. 4J and K).

Several studies indicate that Pea3 transcription factors regulate the expression of different metalloproteinases (MMPs), which are involved in axonal growth of DRG sensory neurons, likely via their known activity on ECM remodeling (Sanz et al., 2017; Yan and Boyd, 2007). Consistent with a role of metalloproteinases in NGF-induced DRG sensory neurite growth, a significant reduction in the neurite length and growth cone area was observed when wild type neurons were treated with the pan-metalloproteinase inhibitor GM6001 (Fig. 5A and B), mimicking the phenotype of *Etv4*$^{-/-}$ neurons. These results indicate that metalloproteinases are required for the correct growth of NGF-dependent embryonic sensory neurons.

Previous evidence suggests that expression of metalloproteinases, such as MMP3, MMP13 and MMP10, as well as urokinase-type plasminogen activator receptor (UPAR), a GPI-anchor molecule that regulates the MMPs activation, are induced by NGF (Chen et al., 2008; Farias-Eisner et al., 2000; Fontanet et al., 2013; Yan and Boyd, 2007). Moreover, the analysis of the promoter sequences of the corresponding genes reveals the existence of specific sites for the binding of PEA3 transcription factors (Bhattacharya et al., 2001; Yan and Boyd, 2007). To analyze the possibility that Etv4 could mediate their expression in response to NGF, we used the neuronal PC12 cell line, which expresses the NGF receptor TrkA and respond to NGF stimulation by increasing Etv4 levels. The cells were

transfected with a plasmid containing a specific *Etv4-shRNA* and stimulated with NGF for 8 h. While an increase in the expression of *Etv4*, *Mmp3*, *Mmp13*, *Mmp10* and *Upar* mRNAs was observed in response to NGF, transfection of PC12 cells with the shRNA for Etv4 inhibited these changes (Fig. 5C). Subsequently, we analyzed the changes in the expression of these molecules in dissociated DRG neuronal cultures obtained from wild-type and *Etv4*-deficient mice, in response to 8h of stimulation with NGF. As shown in Fig. 5D and E, NGF promoted a significant increase in the mRNA expression of *Mmp3*, *Mmp13*, *Mmp10* in DRG neuronal cultures obtained from wild-type but not in cultures from *Etv4*-deficient mice, confirming that Etv4 is required for the induction by NGF of these ECM-remodeling molecules. Although a substantial, but not significant increase in *Upar* mRNA expression was observed upon NGF stimulation, a significant reduction in the levels of this molecule was observed in cultures obtained from *Etv4-KO* mice, indicating that Etv4 is required for promoting *Upar* mRNA expression in NGF-responsive sensory neurons (Fig 5D and E). The absence of the expression of *Etv4* mRNA in DRG neurons obtained from *Etv4*-deficient mice was confirmed by RT-PCR (Fig S4).

In order to analyze whether the deficit in neurite outgrowth observed in *Etv4*$^{-/-}$ neurons is attributable to reduced levels of MMPs, we next asked if exogenously supplied MMPs could revert the phenotype of *Etv4*-deficient DRG cells. To this end, HEK-293 cells were transfected with control or *Mmp3* and *Mmp13*-expressing vectors. After confirming the expression of these molecules (Fig S5), the conditioned medium was added to wild-type and Etv4-deficient DRG neurons cultured in the presence of NGF. Our results indicate that these MMPs were able to compensate the outgrowth deficiency exhibited by *Etv4-KO* neurons, supporting the idea that their induction by Etv4 is necessary for the axonal growth of NGF-dependent sensory neurons (Fig 5F).

Together, these results support a model in which Etv4 would be required for NGF-induced expression of ECM remodeling proteins and for NGF-induced axonal growth. Alteration of this pathway may partially account for the deficiencies in peptidergic nociceptive innervation observed in the epidermis of *Etv4*-mutant mice

Loss of Etv4 results in decreased thermal nociception

Given that Etv4 is highly co-expressed with TrkA during the development of DRG neurons, and is required to mediate NGF/TrkA signaling effect on sensory axonal outgrowth and epidermal innervation, we next asked whether ablation of Etv4 could also affect other NGF-induced sensory properties, such as the sensitivity to painful stimuli. As NGF/TrkA signaling mediates thermal and inflammatory sensory modalities (Khodorova et al., 2017; Ueda et al., 2010), we analyzed the behavioral response of *Etv4*-deficient mice to noxious thermal and chemical stimuli. Hot-plate and tail-flick tests were applied to analyze the responses to acute thermal pain, and the formalin test, as a model of chemically-induced pain.

As heat nociceptors are known to be activated by any temperature above 43ºC, mice were exposed to the hot plate test, subjecting them to a heat stimulus of 48 and 50 ºC, and the withdrawal latency at different temperatures was measured in wild-type and *Etv4*-deficient animals. As shown in Fig. 6A, *Etv4* mutant mice exhibited significantly delayed escape latency at a plate temperature of 50 ºC (but not 48 ºC) compared to wild-type animals, indicating that *Etv4-KO* mice are less sensitive to noxious heat stimuli than wild-type littermates.

Then we used the tail immersion test, in which the tail of each mouse is immersed in water at different temperatures and the time from onset of stimulation to rapid withdrawal of the tail from the water is recorded. Although none of the experimental groups showed differences in response to a water bath temperature of 4ºC or 48ºC, exposure of *Etv4*-deficient mice to 55_ºC caused a significant delay in the withdrawal of the tail from the water compared to wild-type animals (Fig. 6B). Taken together, these results suggest that *Etv4*-mutant animals are less sensitive to noxious heat, normally transduced by peptidergic neurons that express TrkA, but not to noxious cold, which is mediated by Ret-expressing non-peptidergic sensory neurons (Cavanaugh et al., 2009; Lindfors et al., 2006; Lippoldt et al., 2013; Wang et al., 2013).

The formalin test was then applied to examine responses to painful chemical stimuli. Both *Etv4-mutant* and wild-type animals showed two-phase nociceptive responses after intraplantar injection of formalin into the skin of the left hindpaw. No significant differences were detected during the early phase of the test; however, in the second phase, *Etv4*-deficient mice exhibited reduced pain-like responses compared to wild-type

mice (Fig. 6C). Finally, the sensitivity to mechanical stimuli was studied by applying the Von Frey test. No significant differences were observed between the two experimental groups (Fig. 6D).

Overall, we conclude that *Etv4-mutant* animals are less sensitive to noxious heat stimuli and to the second phase of the formalin test, but show normal responses to cold and mechanical stimuli and in the early phase of the formalin test. These findings suggest that Etv4 is required for the nociceptive responses mediated by peptidergic sensory neurons.

Etv4 is required for the proper expression of TRPV1

To gain additional insight on the mechanism through which Etv4 controls nociception, we examined the mRNA expression levels of different receptors and ion channels, including the transient receptor potential (TRP) family of cation channels *TrpV1*, *TrpV2*, *Trpm8*, *TrpA1*, and the voltage-gated sodium channels (VGSCs) *Nav1.7* and *Nav1.8*, all of which being expressed in DRG neurons and known to contribute to the transmission of somatosensory signals (Dib-Hajj et al., 2010). Expression of these genes was analyzed in DRGs obtained from wild-type and *Etv4*-deficient mice at P20. *TrpV1* and *TrpV2* mRNA expression levels were shown to be significantly reduced in *Etv4*-deficient mice, whereas the other molecules analyzed were unaffected. Interestingly, TRPV1 and TRPV2 are known to be associated with pain induced by noxious high temperatures, whereas TRPM8 and TRPA1 detect pain induced by low temperatures (Mickle et al., 2015). No differences were observed in the *Nav1.7* and *Nav1.8* mRNA expression levels, previously linked to the maintenance of inflammatory pain states (Levinson et al., 2012) between wild-type and *Etv4-KO* mice (Fig. 7A).

Considering that TRPV1 is critically involved in the transduction of noxious heat and taking into account that its expression is regulated by NGF (Ji et al., 2002; Zhang et al., 2005), we analyzed possible differences in the number of TRPV1-expressing DRG neurons between *Etv4*-deficient and wild-type mice. A significant reduction in the proportion of TRPV1-positive DRG neurons was detected in *Etv4*-mutant mice (Fig. 7B).

This result raises the possibility that reduction of TRPV1-expressing neurons may result in a loss of TRPV1-dependent peripheral signaling in *Etv4-mutant* mice. To test this hypothesis, we performed the capsaicin test, a well-characterized model of TRPV1–

dependent sensitization of peripheral nerve terminals, using capsaicin, a natural TRPV1 agonist. To this end, capsaicin was injected into the plantar surface of wild-type and *Etv4*-deficient mice, followed by monitoring and quantifying the time spent licking, flinching and guarding (nociceptive behaviors), per minute, for the first 5 minutes thereafter injection. As shown in Fig.7C, both wild-type and *Etv4*-mutant animals spent a similar amount of time developing nociception-associated behaviors during the first 2 minutes after capsaicin injection. However, these nociception-associated behaviors did not persist beyond 3 minutes after injection in *Etv4*-deficient animals, in contrast to controls, in which the behavior pattern was maintained for the rest of the time evaluated (Fig. 7C). Overall, *Etv4-mutant* mice were less sensitive to capsaicin, and this result is consistent with the decreased levels of TRPV1 expression observed in these mice.

Discussion

Our findings point to an essential role of specific transcriptional programs in pain transduction processes. In particular, they suggest that the transcription factor Etv4 plays a crucial role in the development of primary sensory afferent neurons specialized in the transduction of noxious heat and chemogenic inflammatory pain. This molecule participates in the regulation of genes relevant for the transduction of noxious stimuli in the adult. In addition, we found that, while Etv4 is dispensable for the viability of DRG peptidergic neurons, it is involved in the outgrowth and arborization of their projections into the epidermis. Consistently, in vitro assays indicate that Etv4 is required for growth cone expansion and axonal growth of NGF-dependent sensory neurons. From a mechanistic point of view, we show that this process involves the Etv4-mediated regulation of the expression of a group of ECM remodeling enzymes. Moreover, our data indicate that Etv4 is also necessary for the correct maturation of sensory nociceptive neurons expressing TRPV1, and that Etv4 ablation result in the loss of the sensory modality associated with TRPV1 expression.

Based on these data, and our previous work, we propose a model in which, during development, NGF-derived from the target tissue triggers signals that are transported retrogradely along nociceptive axons. These signals promote, during embryonic development, the expression of the Etv4 transcription factor, which in turn, regulates the

expression of effector genes, including those involved in ECM remodeling, which allow an efficient target innervation, maturation of nociceptive neurons, and peripheral pain transduction (Fig. 8).

Pea3 transcription factors in the development of the nervous system

Pea3 transcription factors are expressed during normal development and in oncogenic processes, characterized by extensive remodeling of the ECM (Lu et al., 2009; Oh et al., 2012; Wang and Zhang, 2009). Many studies have emphasized the key role played by Pea3 subfamily proteins in central and peripheral neurons(Chotteau-Lelievre et al., 1997; Hagedorn et al., 2000; Paratore et al., 2002). Etv1 was described in fast-spiking cortical interneurons (Dehorter et al., 2015), Etv4 and Etv5 in hippocampal pyramidal neurons (Fontanet et al., 2018), and Etv1 and Etv4 in spinal cord motoneurons (Arber et al., 2000; Haase et al., 2002; Helmbacher et al., 2003; Lin et al., 1998; Livet et al., 2002; Vrieseling and Arber, 2006). The three Pea3 transcription factors are expressed in different subpopulations of DRG neurons. While Etv1 is found in $TrkC^+$, Etv4 and Etv5 were reported to be expressed in $TrkA^+$ and Ret^+ sensory neurons, and their expression in $TrkB^+$ cells is still unclear (Arber et al., 2000; Fontanet et al., 2013; Patel et al., 2000). These transcription factors are induced by the neurotrophic factors corresponding to each Trk receptor: Etv1 is upregulated in response to NT3 in proprioceptive neurons, while Etv4 and Etv5 are induced in response to NGF in nociceptive cells (Fontanet et al., 2013; Patel et al., 2003). Previously, we have shown that the reduction of Etv4 expression through the use of specific shRNA sequences inhibits axonal growth of sensory neurons in response to NGF, while its overexpression is sufficient to induce axonal growth in response to this neurotrophin (Fontanet et al., 2013). In the present work, we extend these findings, showing that peptidergic DRG and trigeminal sensory neurons from *Etv4*-mutant mice do not respond adequately to NGF.

Etv4 as a cue molecule for peptidergic epidermal innervation

Despite several studies have analyzed the contribution of Etv4 in the CNS its physiological role in the development of the PNS still remains unknown. Previous evidence demonstrated that targeting in the Etv1 locus a transcription factor resulting from the fusion of *Ewing sarcoma (EWS)* with *Etv4* DNA binding domain, results in its

ectopic and precocious expression in postmitotic DRG proprioceptive sensory neurons (which normally do not express Etv4, but express Etv1) and lead to perturbations of axonal projections, the acquisition of differentiation markers and a switch in their dependence on neurotrophic support, indicating that the precise timing and location of ETS transcription factor signaling is essential for normal sensory neuron development (Hippenmeyer et al., 2005). Later, we described, that Etv4, expressed in embryonic DRG peptidergic neurons, plays a role in sensory axonal growth in response to NGF (Fontanet et al., 2013). However, the physiological contribution of this NGF/Etv4 pathway in peptidergic neuron development was not analyzed. Here, we evaluated the physiological consequences of Etv4 depletion in the development of nociceptive sensory neurons. We show that it is necessary for the expression of molecules induced by NGF, that behave as effectors of sensory axonal growth during embryonic development; and is required for correct peptidergic epidermal innervation and pain transduction.

In contrast, Etv4 deficiency did not affected the epidermal innervation of GFRα1/2-positive fibers nor the nociceptive cold behavior, both of which being mediated by non-peptidergic Ret-positive sensory fibers.

Many transcriptional factors have been described as mediators of NGF function in DRG neurons. Although most of them have been associated with the survival and maturation of TrkA-expressing sensory neurons (Huang et al., 2015; Lonze et al., 2002), few have been described as involved in neurite outgrowth and target innervation independently of survival (Harrington and Ginty, 2013). Among them, the serum response factor, SRF, acting downstream of NGF signaling, was shown to promote in vivo axonal growth and target innervation of embryonic DRG sensory neurons, by regulating key cytoskeletal genes (Wickramasinghe et al., 2008).

Previously, we have shown that Etv4 is expressed at high levels in approximately 80% of the TrkA^{+} DRG neurons during a developmental critical period peripheral innervation. Moreover, we have described that the expression of Etv4 in sensory neurons is induced in response to NGF, functioning as a critical mediator of NGF signaling involved in sensory neurite outgrowth (Fontanet et al., 2013). Here, we extend these observations showing that DRG peptidergic neurons in Etv4-null mice have deficient axonal growth and target innervation. Our results indicate that Etv4 is required for the expression of adequate levels of metalloproteinases -such as MMP3, MMP10, MMP13- and UPAR, a

group of molecules involved in ECM remodeling and DRG neurite outgrowth, in response to NGF (Farias-Eisner et al., 2000; Fontanet et al., 2013; Sanz et al., 2017). The promoters of *Mmp3*, *Mmp10* and *Mmp13* characterized by the presence of a binding site for Pea3 factors. The presence of these sites in many *Mmp* promoters makes these genes responsive to different trophic factors (Yan and Boyd, 2007). On the other hand, UPAR is also an important regulator of ECM remodeling and cell signaling. By binding the serine protease urokinase-type plasminogen activator (uPA), UPAR activates plasminogen, generating plasmin, which cleaves different ECM components and activates MMPs. In addition, UPAR is also a signaling receptor that promotes cell motility and invasion (Smith and Marshall, 2010).

Defects in pain sensitivity in Etv4-deficient mice

Different subpopulations of nociceptors are responsible for the encoding of specific nociceptive stimuli. Peptidergic nociceptors have been involved in the transduction of pain induced by thermal and inflammatory stimuli. Our results indicate that Etv4 deletion, while not affecting the number of $TrkA^{+}$ sensory neurons, critically perturbs pain transduction by altering the development of an adequate functional modality of peptidergic epidermal innervation.

At adult stages, peptidergic and non-peptidergic sensory neurons can also be distinguished by the expression of a combination of ion channels involved in the transduction of different external stimuli into electrical activity. While peptidergic neurons have been associated primarily with heat-sensitivity, non-peptidergic neurons play a role in cold detection. The transient receptor potential (TRP) family of ion channels are the main sensors of temperature, cold or heat, and are expressed in different DRG subpopulations (Wang and Siemens, 2015). Among them, TRPV1 was identified as the molecular target of capsaicin (the main active component in hot peppers) and is predominantly expressed in peptidergic neurons (Caterina MJ et al 1999). Genetic ablation of TRPV1 in mice eliminates capsaicin responsiveness and results in an altered reaction to noxious heat stimuli (Caterina et al., 2000). In humans, pharmacological inhibition of TRPV1 reduces their ability to detect painful heat stimuli (Gavva et al., 2008; Rowbotham et al., 2011; Szallasi and Sheta, 2012). When TRPV1 is sensitized in the context of inflammation, it also plays a dominant role in heat detection (Amaya et al.,

2004; Caterina et al., 2000; Caterina et al., 1997; Davis et al., 2000; Huang et al., 2006). Moreover, activation of TRPV1 by intradermally injected capsaicin was shown to induce neurogenic inflammation by promoting the antidromic release of CGRP and substance P (Lin et al., 2007). Furthermore, different studies indicate that the expression, trafficking and phosphorylation of TRPV1 are controlled by the activity of NGF/TrkA (Amaya et al., 2004; Ji et al., 2002; Xue et al., 2007; Zhang et al., 2005). In our study, we found reduced expression levels of *Trpv1* mRNA in DRGs of Etv4$^{-/-}$ animals. Nevertheless, it is not clear how Etv4 would regulate the expression of TRPV1 since Pea3 binding sites have not been described in the promotor of *Trpv1* gene. Interestingly, the transcription factor Sp1 was found to directly regulate TRPV1 in response to NGF, and Etv4 is known to be an interactor of Sp1 (Chu et al., 2011; Jiang et al., 2007). Thus, Etv4 could induce TRPV1 through the formation of a complex with Sp1. Alternatively, Etv4 may induce TRPV1 expression in an indirect manner, by activating expression of other transcription factors acting as relays.

In summary, our data demonstrate that genetic ablation of Etv4 results in reduced responses to noxious heat, formalin, and capsaicin, and suggest that these behavioral phenotypes are the consequence of the lack of a correct peptidergic innervation and/or reduced expression of TRPV1 channels. Although Etv4 is not exclusively expressed in TrkA-positive peptidergic neurons, the absence of behavioral differences between wild-type and *Etv4*-mutant mice exposed to cold or mechanical stimuli, suggests that Etv4 is dispensable for the function of both cold- and mechanoreceptor-sensing nociceptive neurons, characterized by the expression of Ret and TrkB receptors. Indeed, the fact that both, the expression of the Trpv8 cold-sensing channel and the non-peptidergic cutaneous innervation are preserved in Etv4-null mice, additionally supports the idea that Etv4 is not critically involved in the non-peptidergic nociceptive function analyzed here. Taken together, our findings are in accordance with a model in which Etv4 is required for target-secreted NGF-dependent mechanisms that regulate peptidergic axonal growth and the correct maturation of TrkA^{+} nociceptive neurons. Therefore, these results provide a key insight into the transcriptional program by which growth factors regulate axonal growth, innervation, and transduction of sensory pain. Understanding the molecular basis of the perception of noxious stimuli by sensory neurons is essential for the design of new therapeutic treatments for pain.

Material and Methods

Cell lines, plasmids, recombinant proteins and inhibitors

PC12 cells were grown in DMEM in the presence of 5% horse serum and 10% FBS (Invitrogen) (Shirazi Fard et al., 2010). *Etv4*-shRNA, HA-tagged-MMP3 and Flag-tagged-MMP13 vectors were purchased from Cellogenetics. The retroviral vector pRetro-U6G shRNA was used for expression of *Etv4*-shRNA (Fontanet et al., 2013; Fontanet et al., 2018). NGF was purchased from Promega; and the matrix metalloproteinase inhibitor GM6001 was purchased from Calbiochem.

Mouse strain

Etv4$^{-/-}$ mice were kindly provided by Dr. Olivia Bermingham-McDonogh (University of Washington, Seattle, USA). The *Etv4*-mutant mice were described in detail in Livet J et al. (2002). Transgenic strain was genotyped using PCR-based strategy. The use of animals was approved by the Animal Care and Use Committee (CICUAL) of the School of Medicine, University of Buenos Aires, Instituto Leloir and Austral University, according to the Principles for Biomedical Research involving animals of the Council for International Organizations for Medical Sciences and previsions stated in the Guide for the Care and Use of Laboratory Animals.

Sensory neuron culture and neurite outgrowth assay

DRG neurons obtained from *Etv4*$^{+/+}$ and *Etv4*$^{-/-}$mice at embryonic (E) day 14.5 were prepared as previously described (Baudet et al., 2000). Briefly, the ganglia were dissociated with collagenase (2% wt/v, Sigma-Aldrich), trypsin (0.1% wt/v, Invitrogen), and DNAse I (10 µg/ml, Invitrogen). For cranial sensory neuron culture, trigeminal ganglia (TG) from *Etv4*$^{+/+}$ and *Etv4*$^{-/-}$mice at embryonic day 15.5 were collected and dissociated using trypsin (0.05% wt/v, Invitrogen) during 30 min. Cells were seeded onto poly-ornithine (0.5 mg/ml, Sigma-Aldrich) and laminin (10 µg/ml, Sigma-Aldrich) coated 24 well plates at a density of 15000 cells/well and were incubated for 24-36 h. Neurons were maintained in DMEM:F12 (1:1) medium supplemented with 60 mg/ml penicillin, 100 mg/ml streptomycin, 2mM glutamine (Invitrogen), 1 mg/ml BSA (Sigma-Aldrich) (Sensory neuron media). DRG and TG neurons were maintained with NGF (Promega) 50 ng/ml or 10 ng/ml respectively.

Primary cultures of DRG or TG neurons were performed as previously described (see above). Cells were fixed with 4% PFA and stained with anti-βIII-tubulin to identify neurites (mouse anti-βIII-tubulin (cat#: G7121, clone 5G8, dil 1/5,000, Promega)or PFA 4% in the presence of sucrose 4% and stained with anti-βIII-tubulin and Phalloidin (ThermoFisher) to analyze growth cone area. Neuronal survival was evaluated using the nuclear stain 4",6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich). Neurons containing fragmented or condensed nuclear staining were scored as apoptotic cells and not computed in the differentiation assays. Neuron viability was quantified as the number of live neurons in relation to the total amount of neurons (live and apoptotic cells). For DRG dissociated neurons, images were acquired using a Zeiss Observer microscope. For TG dissociated neurons, images were acquired using an Olympus IX-81 inverted microscope. For growth cone analysis, images were acquired using a Zeiss LSM710 NLO confocal microscope (Plan-APOCHROMAT 63X, a.4 N/A). Images were analyzed using the ImageJ software.

Outgrowth assay in the presence of the pan-metalloproteinase inhibitor GM6001 (Calbiochem) was done using 5 μm of the inhibitor. The cells were cultured during 30h and after that, were maintained during 6 h in the presence of GM6001. Cells were fixed with PFA 4% supplemented with sucrose 4% and stained with anti-βIII-tubulin and Phalloidin. All the experiments were done independently at least with 3 mice/condition or genotype in duplicate.

For outgrowth recovery assay with exogenous MMP3 and MMP13, HEK-293T cell line was transfected using polyethylenimine (PEI from Polyscience) with control or HA-tagged-MMP3 and Flag-tagged-MMP13 plasmids, and incubated in sensory neuron media. After 2 days, the conditionate medium containing MMP3 and MMP13 was collected, followed by centrifugation at 20, 000 x g for 20 min. DRG neurons obtained from *Etv4*$^{+/+}$ and *Etv4*$^{-/-}$ mice at embryonic (E) day 14.5 were cultured for 36 h in presence of control or MMP3/MMP13 containing media.

mRNA analysis in PC12 and DRG neurons in culture

PC12 cells were grown in DMEM supplemented with 5% horse serum and 10% FBS (Invitrogen). Cells were transfected using X-treme GENE (Roche) following manufacturer´s instructions. For downregulation of Etv4, PC12 cells were transfected with control or

Etv4-shRNA containing vector and selected with puromycin to enrich in the transfected cells. PC12 *Etv4*-shRNA clones were generated by isolation of colonies transfected with the construct and selected with puromycin as previously described (Fontanet et al., 2013). PC12 control and PC12-*Etv4*-shRNA were stimulated during 8 hours with NGF (50 ng/ml). DRG cultures from *Etv4*$^{+/+}$ and *Etv4*$^{-/-}$ mice at embryonic (E) day 14.5 was performed as previously described (De Vincenti et al., 2021). Neurons were maintained with NGF 20 ng/ml during 44 hours, serum-starved during 12 hours and treated with or without NGF 50 ng/ml for 8 hours.

Immunofluorescence and microscopy

For immunofluorescence assays of DRG and spinal cord, mice at the indicated developmental stages were euthanized and perfused transcardially with 4% paraformaldehyde (PFA) in PBS under deep anesthesia. The lumbar section (L1-L4) containing the DRG and spinal cord was dissected and post-fixed overnight in 4% PFA. DRGs and spinal cord immunostaining were done on 20 µm cryostat transversal sections on glass at the different developmental stages. Tissue sections were permeabilizated for 30 min in PBS containing 0.3% Triton X-100, washed with PBS and then blocked for 1 h with 5% donkey normal serum (DNS) (Jackson ImmunoResearch). Antibodies were applied in PBS containing 0.3% Triton X-100 and 3% DNS. For double labeling with the neuronal marker NeuN and TrkA or TrpV1, a serial protocol was performed. Sections were first permeabilizated for 1 h in PBS containing 1.5% Triton X-100 and incubated with NeuN antibody during 48 h and followed by TrkA or TrpV1 antibody incubation overnight. Secondary antibodies were from Jackson ImmunoResearch.

For neuron quantification or central peptidergic projections quantification assay, images were obtained using an Olympus IX83 DSU 20X objective at a resolution of 2,048 x 2,048 pixels (16 bits) using identical settings between control and experimental images with no saturation. Each image corresponds to a merge of 15 optical sections of 0.9 µm each. The number of CGRP^{+} fibers in the dorsal horn was measured as the total amount of fibers that extend through the lamina I/II into the medial zone of the dorsal horn/section (Hancock et al., 2011). For CGRP intensity levels, corrected CGRP fibers fluorescence was calculated for each dorsal horn of the spinal cord section using a hand-draw ROI. The intensity level was calculated as the integrated density (IntDen) of the selected dorsal

horn area minus the area of the selected dorsal horn times the background (Bck) reading for each image [CGRP intensity level = IntDen – (Area x Bck)]. All images were analyzed using ImageJ software.

For peripheral innervation analysis, 2-month-old mice were euthanized and perfused transcardially with 4% PFA in PBS under deep anesthesia. Glabrous skin from the hindpaw of newborn or 2-month-old mice was sectioned on glass at a thickness of 20 µm. For peptidergic innervation, images were obtained using an Olympus IX83 DSU 20X objective at a resolution of 2,048 x 2,048 pixels (16 bits) using identical settings between control and experimental images with no saturation. Each image corresponds to a merge of 15 optical sections of 0.9 µm each. For non-peptidergic innervation, images were obtained using a Zeiss LSM 880 Airyscan 20X objective (0.8 NA). Each image corresponds to a merge of 12 optical sections of 0.9 µm each. Only fibers crossing the dermis-epidermis line were analyzed on segments of 500 µm of skin using ImageJ software.

Immunofluorescence was performed using the following antibodies: mouse anti-Etv4 (cat#: sc-166629, dil 1/200; Santa Cruz Biotechnology), rabbit anti-NeuN (cat#: D4G40, dil 1/600, Cell Signaling), rat anti-TrkA (cat#: AF1056, dil 1/800 R&D Systems), rabbit anti-TRPV1 (cat#: ACC-030, dil 1/200 Alomone labs), rabbit anti-CGRP (calcitonin gene related peptide cat#: PC205L, dil 1/1000, Sigma-Aldrich), rabbit anti-PGP9.5 (cat#: NE1013, dil 1/1000, Calbiochem), goat polyclonal anti-GFRα1 (cat#: AF560, dil 1/200, R&D Systems), goat polyclonal anti-GFRα2 (cat#: AF429, dil 1/200, R&D Systems).

RT-PCR analysis

For mRNA analysis, total RNA was isolated from lumbar DRG (L1-L6) or DRG cultures, using RNA columns (NucleoSpin RNA) according to manufacturer's instructions and cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen) and random hexamers (Applied Biosystems). The cDNA was amplified using the following primers sets: mouse *Etv4*: forward, 5´-CAG CCC TTT TCC AGG GCA GAA C-3´, reverse, 5´-GCT GGA AGA CGG AGC TGT GCT-3´;mouse *TrkA*: forward, 5´-CTG CCA GGA CAA ACA GAA CAC-3´, reverse, 5´-GTG GTT GGC TTC GTC TGA GTA-3´; mouse *Ret*: forward, 5´-TGA AGA AAA GCA AGG GCC GG-3´, reverse, 5´-ACA ATC TCC CAG AGC AGC AC-3´; mouse *GFRα1*, forward, 5′-GAC CGT CTG GAC TGT GTG AAA G-3′; reverse, 5′- TTA GTG TGC GGT ACT TGG TGC T-3′;rat*Etv4*: forward, 5´-ACT CAG ATG TCC CTG GAT GTG-3´; reverse,

5′-TCA CTC CAT CAC CTG AAG AGG-3′; rat *Mmp3*: forward, 5′-GCT ATT CTG GCC ACC TTC TTC-3′; reverse, 5′-TCA TCA TCA CCA TAG GGA AGG-3′; rat *Mmp13*: forward, 5′-GAC AGA TTC TTC TGG CGT CTG-3′; reverse, 5′-GAA GTT CTG GCC AAA AGG ACT-3′; mouse *TrpV1:* forward, 5′- GAC GGC AAG GAT GAC TTC CG-3′, reverse, 5′- AGT TGC CTG GGT CCT CGT T-3′; mouse *Mmp3*: forward, 5′-TAA AGA CAG GCA CTT TTG GCG-3′, reverse, 5′-TTA GTA ACC TCA TAT GCA GCA TCC A-3′; mouse *Mmp13*: forward, 5′-TGA TGC CAT TAC CAG TCT CC-3′, reverse, 5′-ACA TGG TTG GGA AGT TCT GG-3′; rat and mouse *Mmp10*: forward, 5′-AGG AAG TCA GTT CTG GGC AG-3′, reverse, 5′-GCA TCA ATC TTC TTC ACG GTG G-3′; rat and mouse *Upar*: forward, 5′-AAT GGT GGC CCA GTT CTG GA-3′, reverse, 5′-CCC AGC ACA TCT AAG CCT GT-3′;mouse *Trpv2*: forward, 5′-TTA CTG GGC CAG CTG TGG TA-3′, reverse, 5′-CCT GGG ACA GCA CTG TGA G-3′; *Trpm8*: forward, 5′- AAG AAG TGT TTC AAA TGC TG-3′, reverse, 5′-AAT TCT CCT TCA TGA CAC CC-3′; *Trpa1*: forward, 5′-ATG CAA GAA ACA CGA CAA GA-3′, reverse, 5′-TGA GCT CAT GCT GCT TTT AA-3′; *NaV1.7*: 5′-AAG CTT GGA GGT CAA GAT AT-3′, reverse, 5′-TCA AAA GCT TGG TTT GTC ACT-3′; *NaV1.8*: forward, 5′-CAT GAA GAA GCT GGG CTC CA-3′, reverse, 5′-TGA TGT CAA ATG CTT GCC TGG-3′; rat *Tata binding protein* (*Tbp*): forward, 5′-GGG GAG CTG TGA TGT GAA GT-3′; reverse, 5′-CCA GGA AAT AAT TCT GGC TCA-3′ and mouse *Gapdh*: forward, 5′ TGT TCC TAC CCC CAA TGT GT-3′, reverse, 5′-AGG AGA CAA CCT GGT CCT CA-3′

Behavioral procedures

Mice were acclimatized to the different behavioral testing apparatus at least 2 h during two-three days before the testing day. To measure heat pain, mice were placed within a Plexiglass observation chamber surrounding a hot plate, and the latency to hindpaw flicking, licking or jumping was measured (using a stopwatch). The hot plate was set at two different temperatures, 48 °C and 50 °C and all animals were tested sequentially at each temperature with 90 min between test. For the tail immersion test, the water bath was set at three different temperatures, 4 °C, 48 °C and 55 °C controlled by a digital thermometer. Animals were gently restrained using a restrainer tube, presented previously in the habituation session in order to reduce stress. The tail withdrawal latency was measured (using a stopwatch) by immersing the terminal 3 cm of tail into the water bath. Mice were tested three times at each bath temperature every two minutes with 90 min interval between different temperatures.

To measure responses to chemical nociceptive stimuli, mice were placed in Plexiglass cubicles, and after 30 min, habituation, received an intraplantar injection of 2.5% formalin (30 μl). The amount of time spent licking, flinching and guarding was measured in phases I and II of the formalin test. Phase I included observation during the first 5 min after formalin injection, and phase II run from 10 min to 45 min after formalin injection (Bannon and Malmberg, 2007).

To measure capsaicin-evoked pain, mice were given a 3 μg/10 μl injection of capsaicin (Sigma-Aldrich) in the intraplantar hind leg. The time spent licking, flinching and guarding was measured every 1 min for a period of 5 min after capsaicin injection (Seal et al., 2009).

To measure responses to mechanical stimuli, animals were placed into Plexiglass domes placed on an elevated wire grid. Von Frey nylon monofilaments of different bending forces (0.16, 0.4, 0.6 and 1 g; Stoelting Inc., Wooddale, USA) were used to stimulate the center of the plantar surface of hind legs, following the up-and-down method of Dixon, as described previously (Chaplan et al., 1994). Quantitative assessment of tactile allodynia in the mouse paw was performed to establish the 50% withdrawal threshold. Results were obtained using the formula: 50% g threshold = (10 Xf+kδ)/10.000, where Xf = value (in log units) of the final von Frey hair used; k = tabular value for the pattern of positive/negative responses; and δ = mean difference (in log units) between stimuli.

For all behavioral experiments, only 2-month-old male mice were used to avoid sex difference in nociception.

Statistical analysis

Data were analyzed using GraphPad Prism 8.0 and are expressed as mean ± SEM. Significance was accepted at $p<0.05$. No statistical method was performed in order to define sample size, but our sample sizes were similar to those used in the field and the n of each experiment was indicated in the figure legends. For animal studies the handling of the data was performed in a blinded manner. The selection of the mice was unbiased in terms of size and weight. Statistical analysis was performed in GraphPad Prism 8.0. The normal distribution of the variable was evaluated with the Shapiro-Wilk test. Only for small dataset, normality was assumed. For the statistical analysis of the data, two-tailed

Student's t test, one-way ANOVA or two-way ANOVA analysis followed by a respective post-hoc test was preformed and is indicated in figure legends in each case.

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Conflict of interests

The authors declare no competing or financial interests

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Figures

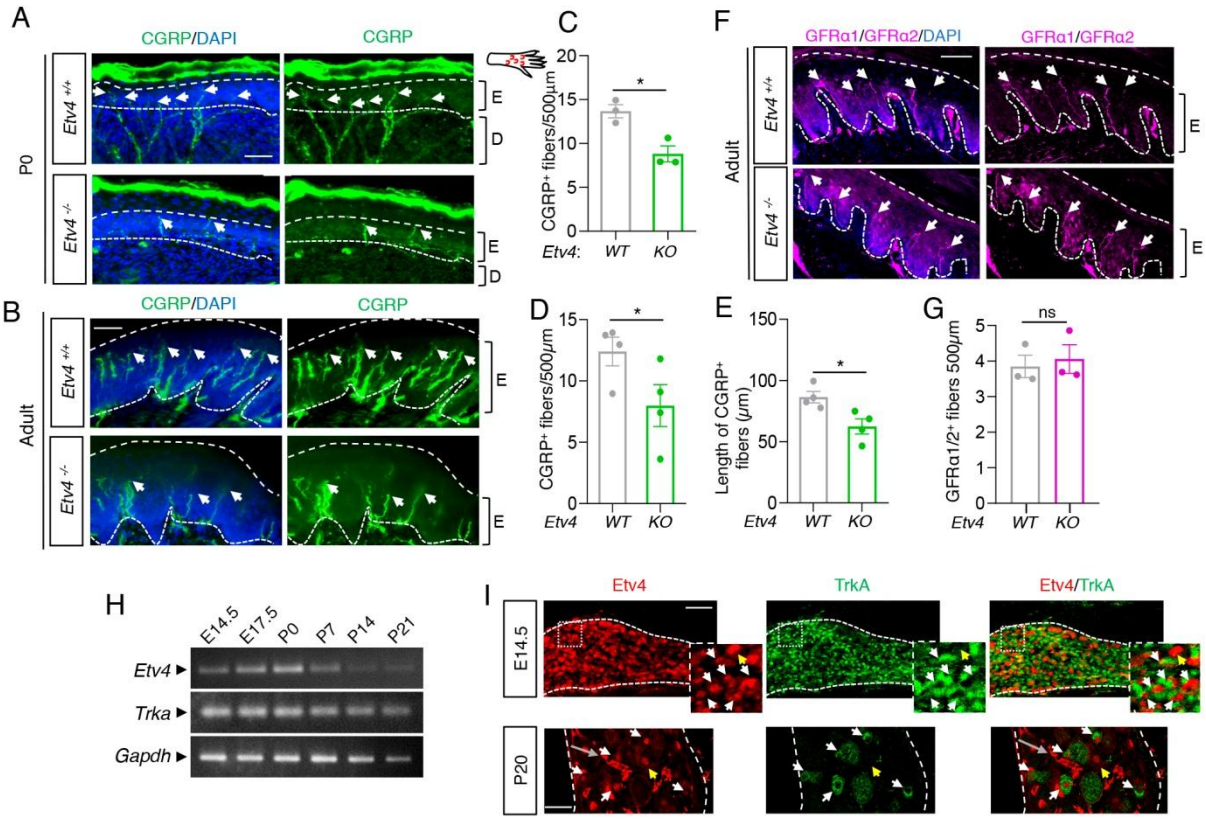

Fig. 1. Developmental expression of Etv4, and epidermal innervation analysis in Etv4 null mice.

(A and B) Images of hindlimb foot pad sections derived from *Etv4*$^{+/+}$ and *Etv4*$^{-/-}$ mice at P0 **(A)** and two-month-old (adult) mice **(B)**, labeled for CGRP to visualize peptidergic projections. Nuclei were stained with DAPI. Dashed lines indicate the limit between dermis (D)-epidermis (E) and epidermis-skin surface. Arrowheads indicate some of the CGRP fibers in the epidermis. Scale bar 50 µm. A scheme of mouse hind paw with the location of the thick glabrous skin indicated in red is shown at the upper corner of panel **A**.

(C and D) The bar graphs show quantification of the number of CGRP$^+$ free nerve endings crossing the dermal-epidermal border per unit length (500 μm) of glabrous skin in control *WT* and *Etv4-KO* mice at P0 **(C)** and adult **(D)** mice. Shown are the mean ± SEM. Number of animals/genotype: for P0, n=3; for adults, n=4. *p<0.05 by two-tail Student´s t test.

(E) The graph shows the quantification of the total length of CGRP$^+$ fibers in adult skin. Shown are the mean ± SEM (n=4 animals/genotype,). *p<0.05 by two-tail Student´s t test.

(F) Images of hindlimb foot pad sections derived from *Etv4*$^{+/+}$ and *Etv4*$^{-/-}$ mice at two-month-old (Adult) mice labeled for GFRα1/GFRα2 to visualize non-peptidergic projections. Nuclei stained with DAPI are shown. Arrowheads indicate some GFRαs$^+$ fibers in the epidermis. Scale bar: 50 μm.

(G) The graph shows the quantification of the number of GFRα1$^+$/GFRα2$^+$ fibers in adult skin per unit of length (500 μm). Shown are the mean±SEM (n=3 animals/genotype). ns denotes not significant by two-tail Student´s t test.

(H and I) Developmental expression and localization of Etv4 in mouse embryonic and postnatal DRG neurons. **(H)** Analysis of developmental expression of *Etv4 and TrkA mRNA* by RT-PCR in DRG. The housekeeping gene *Gapdh* is shown. **(I)** Immunofluorescence in E14.5 (Upper Panel) and P20 (Lower Panel) mouse DRG sections. Upper Panel, scale bar 50 μm. High magnification images of boxed areas are also shown. Lower Panel, scale bar 50 μm. White arrowheads indicate cells showing Etv4-TrkA co-expression. Yellow arrowheads indicate cells expressing Etv4 and negative for TrkA staining. Grey arrow indicates non-specific staining of Etv4.

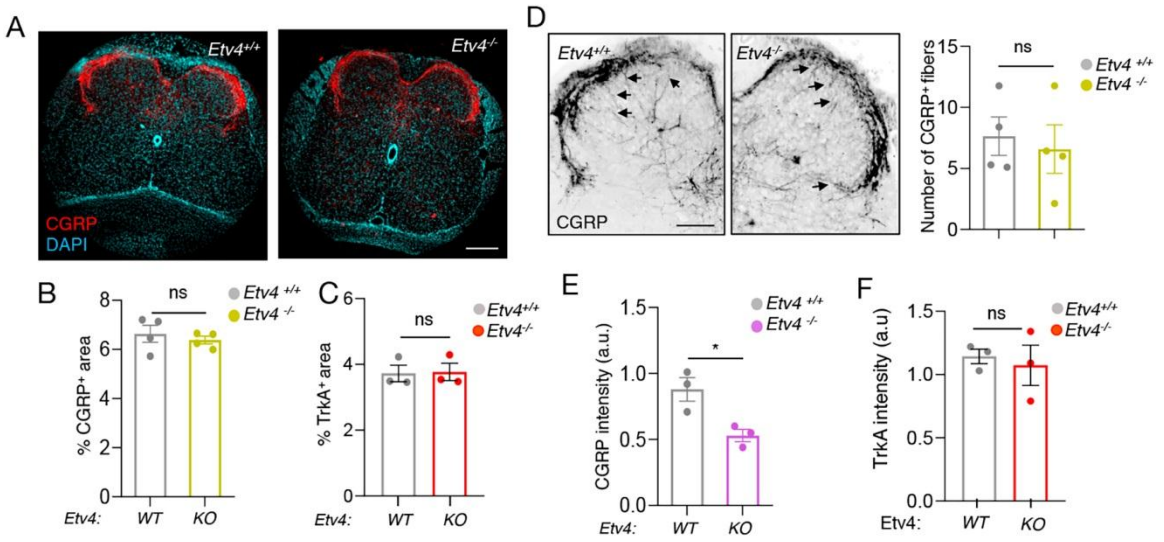

Fig. 2. Analysis of peptidergic central innervation in *Etv4*-deficient

(A) Representative images of spinal cord sections derived from *Etv4*$^{+/+}$ and *Etv4*$^{-/-}$ mice at P15. CGRP^{+} central projections were labeled in red and nuclei were stained with DAPI in cyan. Scale bar 100 µm.

(B-C) The graph shows the percent (%) of the spinal cord area covered by CGRP^{+} **(B)** and TrkA^{+} **(C)** central afferents in superficial laminae of the dorsal horn in spinal cord sections derived from *Etv4*$^{+/+}$ *(WT)* and *Etv4*$^{-/-}$ *(KO)* mice. Data is expressed as mean±SEM of 3-4 mice/genotype. ns denotes not significant by two-tail Student´s t test.

(D) High-magnifications images of the dorsal horn are shown. CGRP^{+} fibers are labeled. Arrows indicate individual CGRP^{+} fibers extended beyond the laminae. Scale bar 50 µm. The graph shows the quantification of the number of CGRP^{+} fibers in spinal cord sections derived from *Etv4*$^{+/+}$ and *Etv4*$^{-/-}$ mice. Data is expressed as mean±SEM of 4 mice/genotype.

(E-F) The graph shows the quantification of the CGRP **(E)** and TrkA **(F)** intensity levels in the dorsal horn normalized to the dorsal horn area expressed as arbitrary units (a.u). Data is presented as mean±SEM of 3 mice/genotype (n=3). *p<0.05 by two-tail Student´s t test.

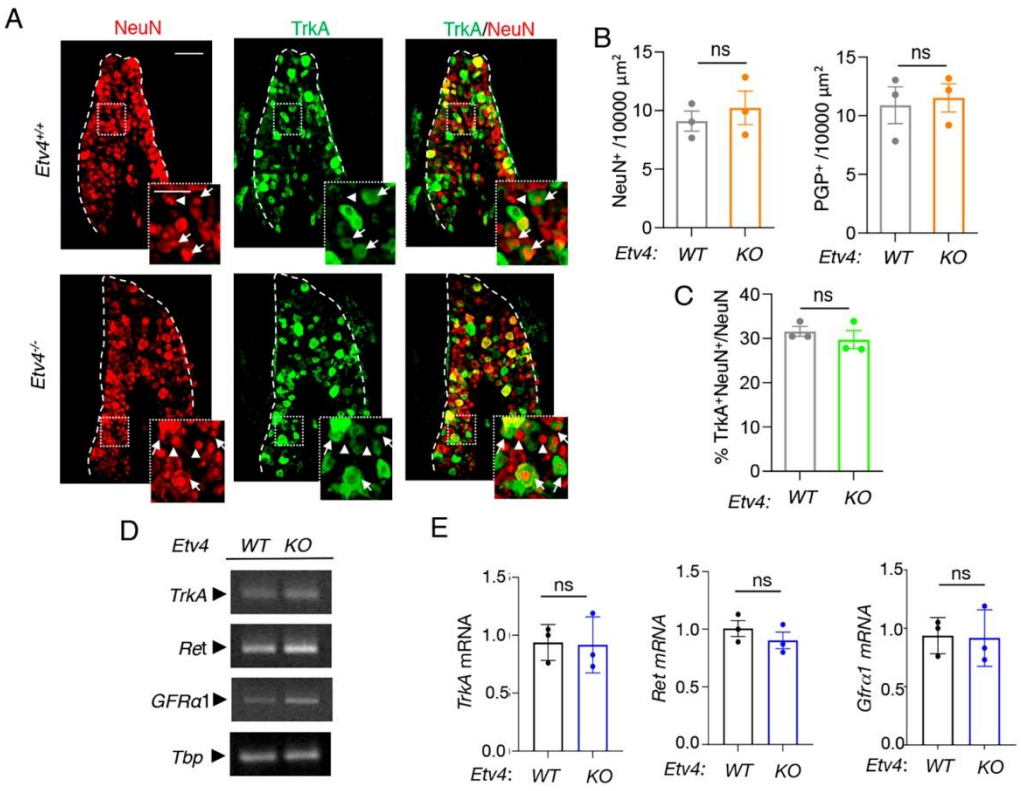

Fig. 3. *Etv4* knockout mice do not show defects in the proportion of TrkA-expressing neurons.

(A) Representative images of DRG lumbar sections expressing the neuronal marker NeuN and TrkA in *Etv4*$^{+/+}$ and *Etv4*$^{-/-}$ mice at P15. Scale bar 100 µm. High-magnifications images of boxed areas are also shown. Arrows indicate individual neurons co-expressing NeuN and TrkA, arrowheads indicate neurons expressing NeuN in the absence of TrkA. Scale bar 50 µm.

(B-C) The graphs describe the density of neurons, NeuN^{+} and PGP9.5^{+} **(B)** cells, in 10000 µm^{2} and the percentage (%) of neurons expressing TrkA respect to the total number of NeuN^{+} cells **(C)** in L4-L5-L6 DRG sections derived from *Etv4*$^{+/+}$*(WT)* and *Etv4*$^{-/-}$*(KO)* mice at postnatal day 15 (P15). The results are expressed as mean±SEM. n=3 mice/genotype. ns denotes not significant by two-tail Student´s t test.

(D-E) Semiquantitative RT-PCR analysis of *TrkA*, *Ret*, *GFRα1* mRNA expression in lumbar DRGs obtained from *WT* and *Etv4-KO* mice at P20 **(D)**. The bar graph shows the levels of mRNA of each molecule normalized to the housekeeping gene *Tbp* **(E*).*** Data is expressed as mean of relative values±SEM, n=3 mice/genotype, ns: denotes not significant (Student´s t test).

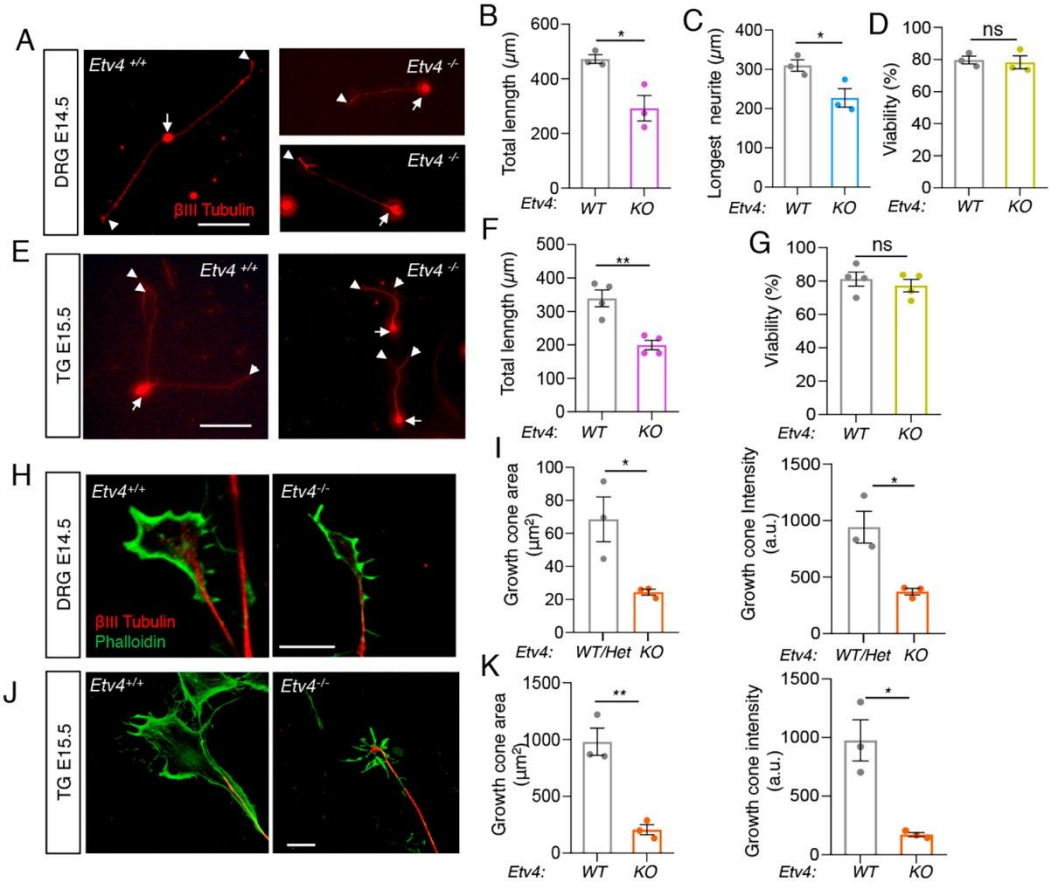

Fig. 4. Etv4 regulates axonal growth and growth cone size in sensory neurons.

(A and E) Representative images show DRG **(A)** or trigeminal ganglion (TG) **(E)** dissociated sensory neurons obtained from *Etv4$^{+/+}$ (WT)* or *Etv4$^{-/-}$ (KO)* mice maintained in the presence of NGF for 24-36 hours and stained with βIII-tubulin. Arrows indicate neuronal cell bodies and arrowheads indicate the neurite tips. Scale bar 100 μm.

(B, C and F) The graphs show the quantification of total neurite length of DRG **(B)** or TG **(F)** sensory neurons and the length of the longest neurite **(C)** of DRG neurons in culture. Results are shown as mean±SEM, DRG sensory neurons from n=3 mice/genotype. TG sensory from 4 mice/genotype were analyzed. *p<0.05, **p<0.005 by Student´s t test.

(D and G) The graphs show the survival of DRG **(D)** and TG **(G)** dissociated sensory neurons. Neuronal survival was evaluated using DAPI for nuclear staining. Neurons containing fragmented or condensed nuclear staining were scored as apoptotic cells. The results are averages±SEM from 3 mice/genotype, ns denotes not significant by two-tail Student´s t test.

(H and J) Representative images of growth cones of DRG and TG from *Etv4*$^{+/+}$ *(WT),* *Etv4*$^{+/-}$ *(Het)* and *Etv4*$^{-/-}$ *(KO)* mice grown in the presence of NGF. Cultures were stained with phalloidin (green) and βIII-tubulin (red). Scale bar: 20 μm.

(I and K) The graphs show the quantification of growth cone area and phalloidin intensity in arbitrary units (a.u.) from DRG **(I)** and TG **(K)** neurons obtained from *WT* and *Etv4-KO* mice cultured in the presence of NGF. For DRG and TG sensory neurons, results are shown as mean±SEM of 3 mice/genotype. *$p<0.05$, **$p<0.005$ by Student´s t test.

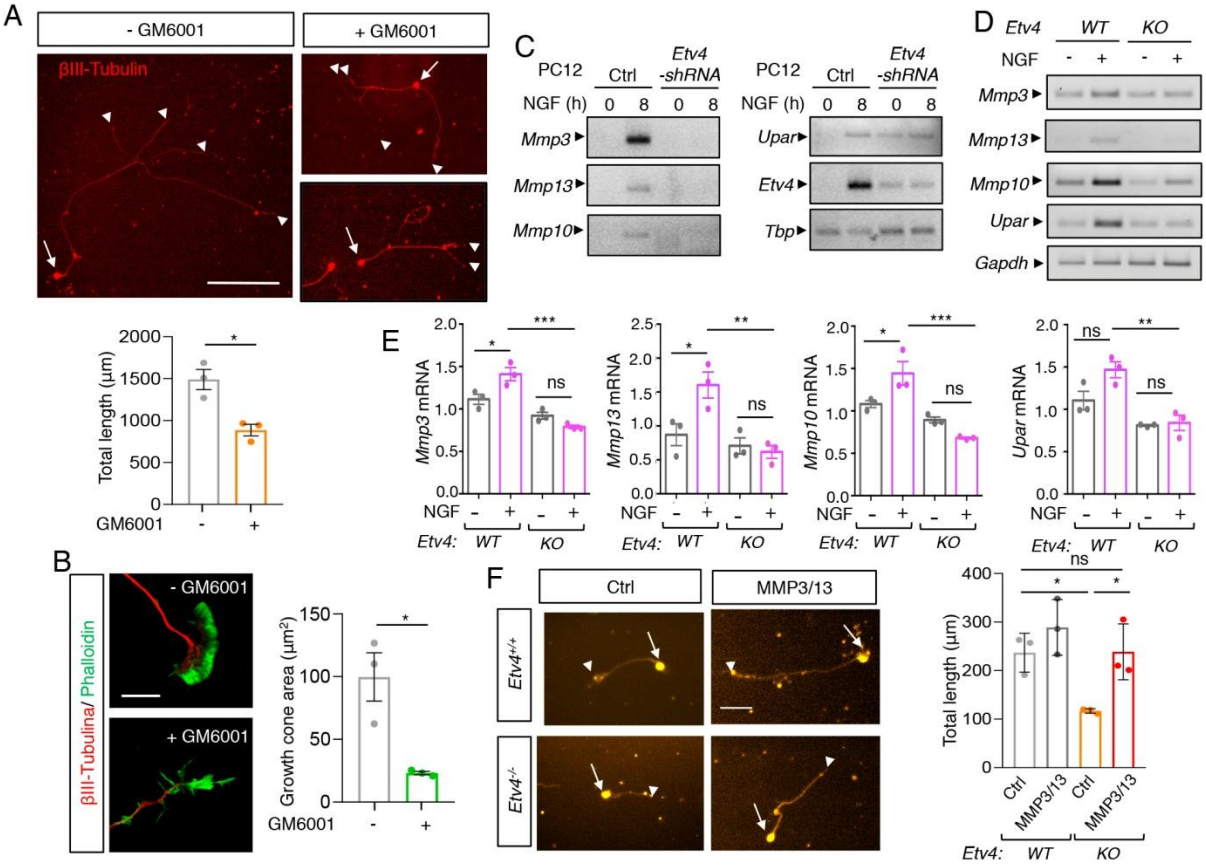

Fig. 5. NGF-induced Etv4 is required for metalloproteinases and UPAR expression involved in neurite outgrowth.

(A) Representative images show DRG neurons cultured in the presence of NGF and treated with the pan-metalloproteinase inhibitor GM6001 (5 μM) or control conditions for 6 hours and stained with βIII-tubulin. Arrows indicate neuronal cell bodies and arrowheads indicate the neurite tips. Scale bar 100 μm. The graph shows the quantification of total neurite length of DRG neurons in culture. Results are shown as mean±SEM, n= 3 animals/ genotype. *p<0.05 by Student´s t test.

(B) Representative images show the growth cone of DRG neurons cultured in the presence of NGF and treated or not with GM6001 (5 μM) for 6 hours and stained with phalloidin and βIII-tubulin. Scale bar: 20 μm. The graph shows the quantification of the

growth cone area. Results are shown as mean±SEM, n=3 mice/genotype. *p<0.05 by Student´s t test.

(C and D) Analysis of *Mmp3*, *Mmp13*, *Mmp10*, *Upar* and *Etv4* mRNA expression by RT-PCR in PC12 transfected with control vector (Ctrl) or *Etv4-shRNA* **(C)** or in sensory neurons derived from *WT* and *KO* mice **(D)** treated with NGF for 8 h. TATA binding protein (*Tbp*) or *Gapdh* was used as a housekeeping gene.

(E) Bar graphs show the analysis of *Mmp3*, *Mmp13*, *Mmp10* and *Upar* mRNA levels from sensory neurons derived from *Etv4 WT* or *KO* mice treated with NGF for 8 h analyzed by semiquantitative RT-PCR. The levels of mRNA were normalized using the expression of the housekeeping *Tbp*. Data from an assay performed in triplicate is expressed as mean of relative values±SEM. 6 mice/genotype were used, *p<0.05, **p<0.01, ***p<0.001 by ANOVA followed by Tukey´s multiple comparison test.

(F) Representative images show DRG neurons obtained from *WT* or *Etv4-KO* mice cultured in the presence of medium conditioned by HEK-293 cells transfected with control (Ctrl) or plasmids expressing MMP3 and MMP13 (MMP3/13) in the presence of NGF. Arrows indicate neuronal cell bodies and arrowheads indicate the neurite tips. Scale bar: 100 µm. The graph shows the quantification of total neurite length of DRG neurons. Results are shown as mean ± SEM, 3 animals/genotype were analyzed, *p<0.05, ns denotes not significant by Two-way ANOVA followed by Tukey´s multiple comparisons test.

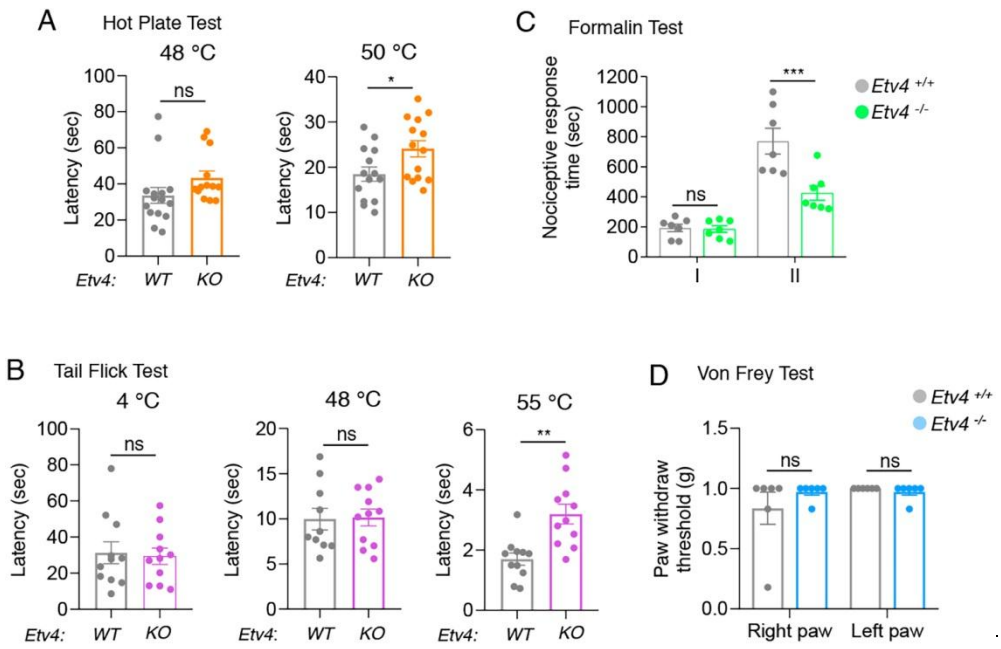

Fig. 6. Etv4 knockout mice are less responsive to noxious heat sensation and inflammatory pain.

(A-B) Thermal sensitivity of *WT* and *Etv4-KO* mice evaluated in hot plate test **(A)** and water tail flick test **(B)** at the indicated temperatures. The graphs show the latency (seconds indicated as sec) it takes for the animal to lift or lick the paw **(A)** or the latency (seconds) of tail withdrawal when it was immersed in a beaker of water **(B)**. The results are expressed as mean±SEM. *p<0.05; **p<0.01 by two-tail Student´s t test. ns denotes not significant. Number of animals analyzed of each genotype in hot plate test: *WT* (n=15), *Etv4-KO* (n=14),

(C) The bar graph shows the inflammatory pain induced by the intraplantar formalin injection in *Etv4-KO* compared to *WT* mice. Shown are the nociceptive behaviors scored as the amount of time spent (sec.) in nociceptive response in the injected hind paw; PHASE I: 0–5 min, PHASE II: 10-45 min after formalin injection. Results are presented as

mean±SEM. n=7 mice/genotype. ns denotes not significant, ***p<0.001, by two-way ANOVA followed by Bonferroni multiple comparisons test**.**

(D) Responses to mechanical stimulation using the Von Frey test measured as described in Materials and Methods. Results are presented as mean±SEM. n=6 mice/genotype. ns denotes not significant by two-way ANOVA followed by Bonferroni multiple comparisons test.

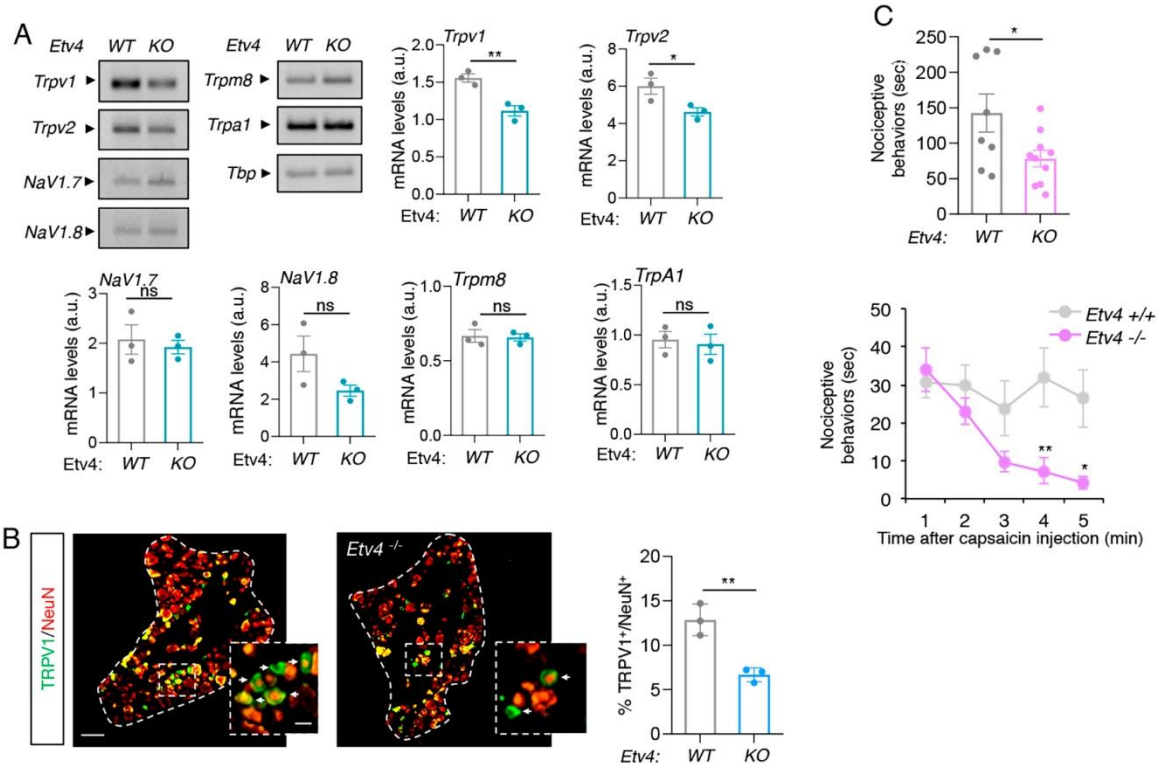

Fig. 7. Etv4 deletion results in a reduction of TRPV1 sensory neurons *in vivo* and in hyposensitivity to pain induced by capsaicin.

(A) Semiquantitative RT-PCR analysis of *TrpV1, TrpV2, Trpm8, TrpA1, Nav1.7 and Nav1.8* expression in lumbar DRGs obtained from *WT* and *Etv4-KO* mice at P20. The bar graphs show the levels of mRNA normalized using the expression of the housekeeping gene *Tbp*. a.u. indicates arbitrary units. Data is expressed as mean±SEM., n=3 mice/genotype,**p<0.01 *p<0.05; by Student´s t test. ns: denotes not significant (Student´s t test).

(B) Representative images show TRPV1$^+$ neurons in L4-L5-L6 DRG sections from *WT* and *Etv4-KO* mice at P20. Neurons stained with the neuronal marker NeuN are shown. Scale bar 100 µm. High-magnifications images of boxed areas are shown. Arrows indicate individual neurons co-expressing NeuN and TRPV1. Scale bar 50 µm. The graph

describes the proportion of $NeuN^+$ neurons expressing TRPV1 in *WT* and *Etv4-KO* mice. Data is expressed as mean±SEM of 3 mice/genotype. **p<0.01 by two-tail Student´s t test.

(C) The upper graph shows the time (sec) spent by mice in nociceptive behaviors after intraplantar capsaicin injection. Data is expressed as mean ± SEM. *WT*: n=8 and *Etv4-KO*: n=10, *p<0.05; two-tailed Student´s t test. The lower graph shows the time course of the assay discriminating the nociceptive behaviors displayed at each time point. Data is expressed as mean±SEM. *p<0.05, **p<0.05 by two-way ANOVA followed by Bonferroni multiple comparisons test.

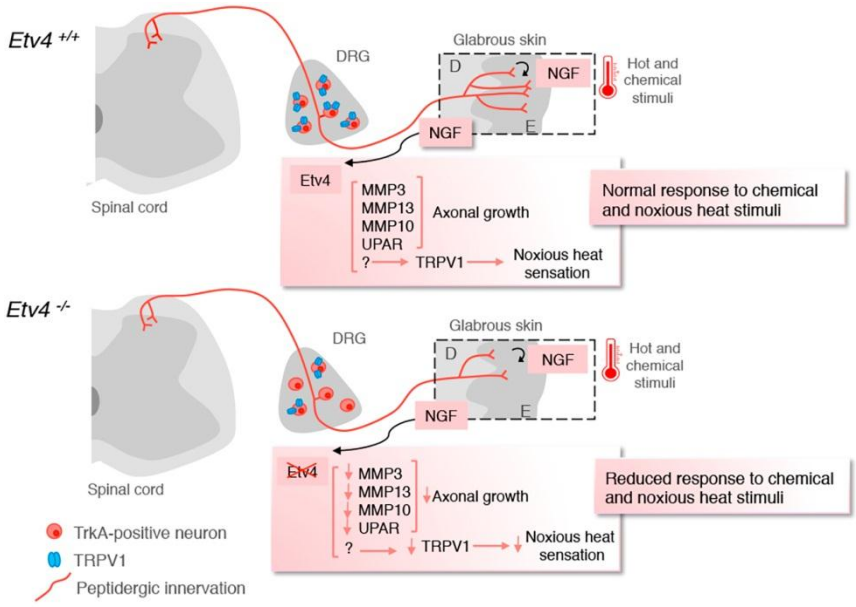

Fig. 8. Model summarizing the effects of *Etv4*$^{-/-}$ on peripheral sensory innervation and DRG nociceptive behavior.

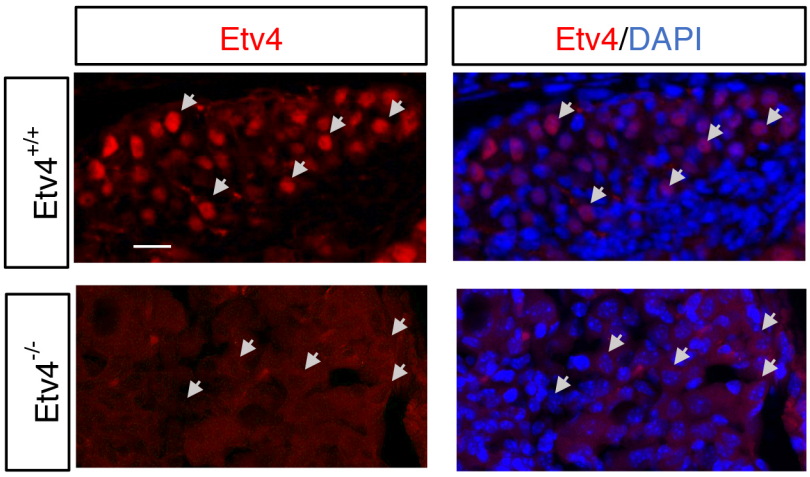

Fig. S1. Control of anti-Etv4 antibody specificity

Images of DRG sections obtained from newborn Etv4$^{+/+}$ and Etv4$^{-/-}$ mice stained with anti Etv4 (red) antibodies and the nuclear staining DAPI (blue). Scale bar: 25 µm. Arrowheads indicate neuronal nucleus

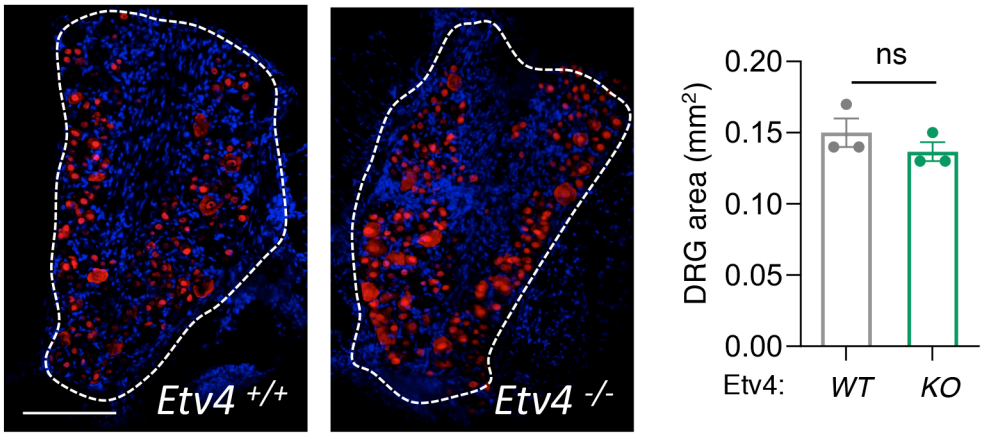

Fig. S2. Etv4 is not required for DRG sensory neurons survival

Representative images show the comparable size of L4-L5-L6 DRG from *Etv4*$^{+/+}$ and Etv4 -/- mice at P15. Neurons were stained with the neuronal marker NeuN (red) and nuclei were stained with DAPI (blue). Scale bar 500 µm. The graph describes the area (mm2) of L4-L5-L6 DRG sections derived from *Etv4*$^{+/+}$ and *Etv4*$^{-/-}$ mice. Data is expressed as mean ± SEM of 3 mice/genotype (n=3, 13 sections/animal). ns denotes not significant by two-tailed Student's t test. p=0.3295.

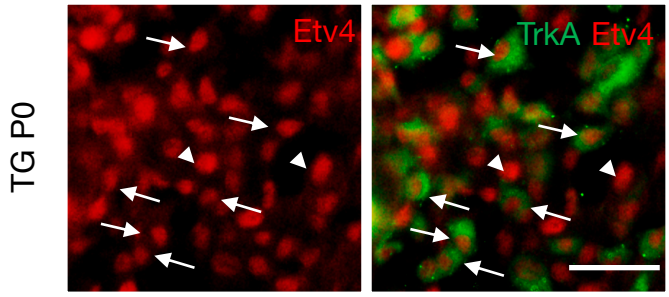

Fig. S3. Etv4 is expressed in TrkA-positive trigeminal sensory neurons

Representative images of trigeminal ganglia (TG) coronal sections showing the expression of Etv4 (red) and TrkA (green) at P0. Arrows indicate individual neurons coexpressing Etv4 and TrkA, arrowheads indicate neurons expressing Etv4 in the absence of TrkA. Scale bar 50 µm.

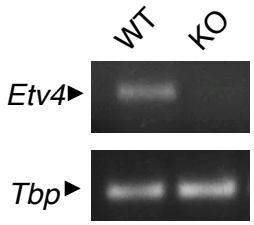

Fig. S4. Control of *Etv4* mRNA expression in DRG from control or Etv4-deficient mice used to analyze MMPs and Upar expression. TATA binding protein (Tbp) was used as housekeeping gen

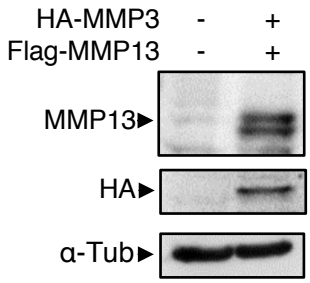

Fig. S5. Control of MMP3 and MMP13 expression in HEK-293 cells

HEK-293 cells were transfected with control or MMP3-HA and MMP13-Flag expressing plasmids. Cell extracts were analyzed by immunoblot to show expression of the different MMPs by using antibodies against HA or MMP13. The same blot was probed for α-Tubulin.

Supplementary Methods and Reagents

Western Blotting of MMPs

HEK-193 cells were lysed at 4°C in lysis buffer containing 0.5% Triton X-100, 0.1% SDS plus protease, and phosphatase inhibitors. Protein lysates were clarified by centrifugation and analyzed by immunoblotting as previously described (Fontanet et. al 2013). The blots were scanned in a Storm 845 PhosphorImager (GE Healthcare Life Sciences). For western-blot, the following antibodies were used: rat monoclonal anti-HA (3F10, dil. 1/1000, Roche), rabbit polyclonal anti-MMP13 (sc-30073, dil. 1/500), mouse anti-α tubulin (cat# T9026, Sigma, dil. 1/6000).