Transcription factor mesenchyme homeobox protein 2 (MEOX2) modulates nociceptor function

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Mesenchyme homeobox protein 2 (MEOX2) is a transcription factor involved in mesoderm differentiation, including development of bones, muscles, vasculature and dermatomes. We have previously identified dysregulation of MEOX2 in fibroblasts from Congenital Insensitivity to Pain patients, and confirmed that htn, the Drosophila homologue of MEOX2, plays a role in nocifensive responses to noxious heat stimuli. To determine the importance of MEOX2 in the mammalian peripheral nervous system, we used a Meox2 heterozygous (Meox2+/−) mouse model to characterise its function in the sensory nervous system, and more specifically, in nociception. MEOX2 is expressed in the mouse dorsal root ganglia (DRG) and spinal cord, and localises in the nuclei of a subset of sensory neurons. Functional studies of the mouse model, including behavioural, cellular and electrophysiological analyses, showed altered nociception encompassing impaired action potential initiation upon depolarisation. Mechanistically, we noted decreased expression of Scn9a and Scn11a genes encoding Na+,7
Introduction

Mesenchyme homeobox protein 2 (MEOX2, also known as growth arrest specific homeobox protein, Gax or MOX-2) gene encodes for the transcription factor MEOX2, which is expressed in the paraxial mesoderm as early as E8.5-9 and is critical for mammalian muscle and bone development as well as vascular differentiation [1–3]. By regulating myogenic genes such as Pax3 and Myf5, it plays a role in development of limb musculature [4]. Meox2−/− (knock-out, KO) mice are either embryonically lethal or die before weaning, have severe limb defects, a cleft palate, reductio of overall skeletal muscle mass and/or complete absence of certain muscles [4,5]. Reduction of MEOX2 expression has been shown in brains of Alzheimer’s disease patients [6], and its haploinsufficiency was reported to lead to neurovascular dysfunction and susceptibility to Amyloid-β toxicity in Meox2−/− mice [7]. MEOX2 mRNA dysregulation was reported in fibroblasts derived from Congenital Insensitivity to Pain (CIP) patients from two unrelated families with mutations D31Y and E172D in the transcription factor PR (PRDI-BF1 and RIZ homology) domain containing member 12 (PRDM12) [8]. CIP is a rare genetic disorder affecting the survival or function of nociceptors, a specialised set of sensory neurons that detect pain, rendering patients unable to feel painful or noxious stimuli [9]. In the fly model, Drosophila melanogaster sensory neuron specific ablation of buttonless (bin, the fly homologue of MEOX2) results in the reduction of nociceptive behaviour in fly larvae in response to noxious heat stimulation. This suggests an important role of MEOX2 in sensory neuron function pertaining to pain [8]. However, mechanistic insight into the role of MEOX2 in the function of the mammalian sensory nervous system is unavailable.

Nociceptors are primary afferent neurons whose somata reside in dorsal root ganglia (DRG). They maintain a transcriptional programme that enables expression of specialised gene products designed for the transduction, transformation and synaptic transmission of noxious stimuli. Temperature and chemical irritant transducers expressed in nociceptors include transient receptor potential (TRP) cation channels, including capsaicin-sensitive TRPV1 [10], and TRPA1, which is responsive to noxious heat, cold and electrophilic chemicals [11,12] and/or heat-sensitive voltage-gated Na+ channels, for example, Na+,1.7, Na+,1.8 and Na+,1.9 [13]. Nociceptor nerve endings densely innervate dermal and epidermal layers of the skin, internal surfaces and organs including gut and bone. Nociceptors integrate painful information and synapse with neurons of the central nervous system in lamina I, II and V of the spinal dorsal horn from where spinothalamic and spinoreticular tracts project to the brain.

We characterised the function of MEOX2 in sensory neurons, by analysing MEOX2-deficient mice. In our hands, classic Meox2 KO mice (Meox2−/−) were neonatally lethal, therefore we performed our analyses on Meox2 haploinsufficient mutant mice, Meox2−/− (MORE, Mox-2Cre) [5].

Results

To determine the expression of MEOX2 in wild-type (WT) adult mouse tissue, we performed Western blotting analysis using specific antibodies against MEOX2 and noted its expression at variable levels in different tissues, such as kidney, lung and most notably liver and heart (Fig. 1A). Additionally, we noted MEOX2 to be highly expressed in both peripheral and central nervous systems, including DRG, spinal cord, cerebellum, hippocampus, hypothalamus and cortex (Fig. 1B). Immunohistochemical double labelling of DRG sections harvested from WT mice using anti-MEOX2 antibodies together with sensory neuron markers, specifically antibodies against calcitonin gene-related peptide (CGRP), tropomyosin receptor kinase A (TrkA), Na+,1.8 or isolectin B4 (IB4), demonstrated that MEOX2 co-expressed with markers for polymodal nociceptors (Fig. 1C–F). It was also present in neurons expressing the nociceptor-specific sodium channel Na+,1.8 (Fig. 1E) and IB4 (Fig. 1F).
To address the role of MEOX2 in nociception we analysed Meox2 haploinsufficient animals described previously [5]. We first confirmed the reduction of Meox2 mRNA by RT-qPCR and MEOX2 protein level by Western blot in Meox2−/− mice and control littermate DRG (Fig. 2A,B). Considering the critical role for MEOX2 in limb muscle development, we confirmed that Meox2−/− had no limb muscle defects by analysing their performance on an accelerating Rotarod, an assay used to test endurance, balance, grip strength and locomotion [23]. The performance of Meox2−/− on the accelerating Rotarod revealed no

Fig. 1. Mesenchyme homeobox protein 2 (MEOX2) is expressed in both central and peripheral nervous tissues. (A) Western blot analysis of wild-type tissue shows relatively ubiquitous but variable expression levels of MEOX2 in kidney, liver, lung and heart, and (B) throughout the peripheral and central nervous systems; DRG – dorsal root ganglion, SC – spinal cord; CBL – cerebellum; Hipp. – hippocampus; Hypo. – hypothalamus; WB – whole brain, n = 1. (C) Representative sections of adult wild-type lumbar DRG co-immunolabelled with antibodies against MEOX2 (green) and CGRP (red); (D) TrkA (red), (E) Na1.8 (red) and (F) IB4 (red). DAPI nuclear staining in blue for all panels; white arrowheads indicate double positive cells rightmost merged image for all. For CGRP, TrkA and IB4 staining, n = 3 WT; 2-3 sections per 5 DRG/animal; for Nav1.8 staining n = 2 WT; 2 section per 5 DRG/animal. Quantification of colocalisation on the right, expressed as percentage of single or double stained DAPI+ cells. Scale bar is 20 µm.
differences as compared to their littermate controls (Fig. 2C). We next examined the organisation of superficial spinal cord dorsal horn Rexed laminae by immunofluorescence staining of IB4 and PKCγ, markers of laminae I and II respectively. Previous studies have implicated other homeobox containing transcription factors to organise the topographical patterning of the laminae where nociceptor projections terminate, particularly IB4-expressing lamina I [24,25]. However, there were no notable disruptions in the laminae I and II patterning or optical density changes of IB4 and PKCγ immunoreactivity in Meox2+/− spinal cord sections as compared to littermate controls (Fig. 2D). Finally, we determined that the paw skin of Meox2+/− was innervated by peripheral nociceptor endings required to detect noxious stimuli as revealed by pan-neuronal PGP9.5 immunoreactivity (Fig. 2E). Together these data suggest that MEOX2 haploinsufficiency did not cause major hind limb muscular deficiencies, nociceptor morphological alterations or their innervation patterns in the spinal cord.
Fig. 2. Meox2+/− mice exhibit impaired behavioural responses to acute and inflammatory pain stimuli. (A) Levels of Meox2 mRNA in Meox2+/− (red bars) compared to Meox2+/+ littermate DRG (black bars) relative to Gapdh. Levels of mRNA are normalised to the average of Meox2+/− samples. n = 3 WT and 3 HET, mean ± SEM; Unpaired Student’s t-test, P-value * < 0.05, ** < 0.01 or as indicated. (B) Western blot showing decreased MEOX2 expression levels in Meox2+/− DRG as compared to Meox2+/+ littermate DRG. SC-Spinal cord. GAPDH is used as a loading control. MW molecular weight marker, n = 1 animal per genotype. (C) No difference in the latency to fall off the accelerating Rotarod of Meox2+/− and Meox2+/+ littersmates; n = 7 WT and n = 12 HET, mean values ± SEM; two-way ANOVA with Sidak’s multiple comparisons test. (D) Representative immunolabelling of the lumbar dorsal spinal cord sections of adult Meox2+/− (top row) and Meox2+/+ (bottom row) littermates, with anti-IB4 (red) and −PKCV (green) antibodies. Rightmost panels are merged red and green channels, with immunoreactive laminae labelled. Scale bars = 50 μm. Quantification of relative optical density (ROD), bottom, of IB4 or PKCV immunoreactive terminals normalised to mean of control. n = 2 WT and 2 HET; dorsal horns from each side (when possible) from a total of 36 spinal cord sections were counted; data represents mean ± SEM. There are no evident patterning alterations or ROD changes in the IB4 or PKCV labelled laminae in the Meox2+/− spinal cord sections as compared to controls. (E) Representative immunofluorescent staining of at least three animals per genotype of FG9.5 (red) in hind paw planar skin sections of Meox2+/− and Meox2+/+ littermate control. White arrow heads indicate nerve fibres in the epidermis; scale bar 20 μm. (F) The latency to first reaction following placement on 50 °C or 52 °C hot plate (left panel); n = 36 WT and 27 HET; mean values ± SEM; unpaired Student’s t-test; P-value * ≤ 0.05, ** ≤ 0.01 as compared to littermate controls. Finally, we performed the formalin test to invoke inflammatory pain by intraplantar hind paw injection of 2.5% formalin, and observing the reaction of the affected paw for 50 min [26]. The two main phases of the formalin test, phase I (0–10 min) and phase II (11–50 min), reflect acute pain responses associated with nociceptor activation in phase I, and inflammatory pain processes as well as spinal sensitisation during phase II [26,27]. Pain-related behaviour in response to formalin was significantly less pronounced in phase II in Meox2+/− animals as compared to littermate controls (Fig. 2G).

We then subjected the animals to acute heat pain by using the hot plate test. The reaction latencies of Meox2+/− mice to 50 °C were similar to their littermate controls, however, at 52 °C reaction latencies were significantly prolonged (Meox2+/− mean latency 16.84 s ± 1.049 vs. Meox2+/+ 20.82 s ± 1.176; Fig. 2F, left panel), suggesting higher pain thresholds to noxious heat stimuli than their WT counterparts. Hind-paw intraplantar injection of TRPV1 agonist, capsaicin, evoked significantly shorter reaction times in Meox2+/− animals as measured by licking, biting, shaking and lifting of the injected paw (Meox2+/− mean reaction time 32.92 s ± 3.43 vs. Meox2+/+ 20.5 ± 2.76; Fig. 2F, right panel), confirming a higher pain tolerance when compared to littermate controls. Finally, we performed the formalin test to invoke inflammatory pain by intraplantar hind paw injection of 2.5% formalin, and observing the reaction of the affected paw for 50 min [26]. The two main phases of the formalin test, phase I (0–10 min) and phase II (11–50 min), reflect acute pain responses associated with nociceptor activation in phase I, and inflammatory pain processes as well as spinal sensitisation during phase II [26,27]. Pain-related behaviour in response to formalin was significantly less pronounced in phase II in Meox2+/− animals as compared to littermate controls (Fig. 2G).

To address the observed behavioural deficiencies in MEOX2 haploinsufficiency on a cellular level, we performed intracellular Ca2+ measurements in cultured DRG neurons that were stimulated with specific nociceptor agonists, including TRPV1 agonist capsaicin, MrgrprA3 agonist chloroquine, TRPA1 agonist PF-4840154 and TRPM8 agonist WS12. The capacity of sensory neurons to respond to the respective chemical stimuli was measured as ratiometric fluorescence signals of the Fura-2 Ca2+ indicator dye in 1365 Meox2+/− cultured DRG neurons and 1618 Meox2+/+ littermate control neurons. Cytoplasmatic Ca2+ transients upon stimulation were similar in both genotypes for capsaicin and with KCl as a positive control; however, voltage-gated potassium channel blocker tetraethylammonium (TEA) slightly increased Ca2+ transients in Meox2+/− as compared to controls (Fig. 3A,B). This indicated no impairment of capsaicin responses in Meox2+/− cultured neurons. Interestingly, we found substantially increased responses to MrgrprA3 agonist chloroquine and TRPA1 agonist PF-4840154 in Meox2+/− cultured neurons compared to littermate control cells (Fig. 3C,D). To determine if the behavioural phenotype in Meox2+/− animals translates to observable electrophysiological properties changes, we examined the role of MEOX2 in excitability of cultured sensory neurons from Meox2+/− mice and littermate controls. First, analysis of voltage-gated currents evoked by 5 mV depolarising pulses, revealed no significant changes in either outward or inward currents (Fig. 4A,B). Additionally, when individual I/V-plots were modelled to determine maximal conductance density (Gmax), activation voltage and slope of activation, no difference was observed between phenotypes (Fig. 4A). Action potential characteristics of sensory neurons derived from Meox2+/− and their littermate controls were indistinguishable (Fig. 4C,D). Sensory neurons were challenged with a thermal stimulus to determine whether they
**Fig. 3.** Calcium imaging responses as measured by ratiometric fluorescence intensity upon stimulation of Meox2+/− cultured sensory neurons. (A) Calcium imaging fluorescence intensity traces upon 30 s stimulation with 1 µmol-L−1 capsaicin, 3 mmol-L−1 TEA and 60 mmol-L−1 KCl of 1365 Meox2+/− (red trace) and 1618 Meox2+/− (black trace) neurons. Traces represent F340/F385 nm ratio of the corresponding stimulation wavelengths of Fura-2 dye. (B) Area under the curve measured for the duration of the stimuli were similar in Meox2+/− and Meox2+/− cultured neurons. (C) Time course of cytosolic calcium upon 30 s stimulation with 100 µmol-L−1 chloroquine, 500 mmol-L−1 WS12 and 1 µmol-L−1 PF4941054 of 1365 Meox2+/− (red trace) and 1618 Meox2+/− neurons (black trace). Traces represent the F340/F385 nm ratio of Fura-2 loaded neurons. (D) Area under the curve measured for the duration of the stimulation for corresponding traces from C. n = 2 WT and 2 HET with at least six technical replicates; data are mean ± SEM; unpaired Student’s t-test; P-value ** ≤ 0.01; **** ≤ 0.0001, or as indicated.

**Fig. 4.** Summary of the electrophysiological characteristics of cultured Meox2+/− and littermate dorsal root ganglia (DRG) cultures. (A) Voltage-gated currents evoked by 5 mV depolarising pulses of Meox2+/− DRG cultures (n = 72 cells) and littermate controls (n = 37 cells) showing inward peak currents, left panel and parameters derived from inward peak currents, right panel (maximal conductance density (G), activation voltage (Vact) and slope of activation, as labelled), reveal no difference was observed between phenotypes. (B) Outward peak voltage-gated currents evoked by 5 mV, left panel, and outward sustained current, right panel of Meox2+/− DRG cultures (n = 72 cells) and littermate controls again (n = 37 cells), show no difference. For all panels, Meox2+/− black and Meox2+/− red traces, as labelled. (C) Representative evoked action potential by current injection recorded from Meox2+/− derived sensory neurons (red trace) compared to action potentials derived from their corresponding littermate controls (black trace). (D) Table of evoked action potential characteristics. Capacitance of the recorded DRG, reflecting the amount of plasma membrane (PM); Rmem; permeability of the PM in voltage clamp at −60 mV; Vmem; CCclamp; resting membrane potential of non-firing DRG in current clamp recording; Rinp; input resistance, permeability of the PM in current clamp; IAP; minimal current injection (50ms duration) to evoke a single action potential; VAP; resting membrane potential during IAP recordings, prior to the current injection; OS; overshoot of the action potential, maximal voltage recorded; US; undershoot of the action potential, minimal voltage recorded. AP Thres; threshold, minimal membrane voltage at which an action potential will/can be generated; dV/dtmax; maximal speed of depolarisation; dV/dtmax1; maximal repolarisation speed in the first phase of repolarisation; dV/dtmax2; maximal repolarisation speed in the second phase of repolarisation; t1−t2: time in ms between dV/dtmax and dV/dtmax1; t1−t3: time in ms between dV/dtmax and dV/dtmax2. The afterhyperpolarisation, is fitted with a single exponential function where t1 describes the speed of recovery exp. A0 + A1(1−t/exp(t1)). DRG cultures were harvested from two WT and four HET animals.
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A

B

C

D

General characteristics

| MEOX2+/+ (N = 26) | MEOX2+/– (N = 72) | P (t-test) |
|-------------------|-------------------|-----------|
| Capacitance (pF)  | 24.57 ± 1.10      | 24.43 ± 1.37 | n.s. |
| R_series (MΩ)     | 7.55 ± 0.44       | 7.73 ± 0.25  | n.s. |
| V_memb Clamp (mV) | –49.51 ± 0.90     | –51.06 ± 0.74 | n.s. |
| R_input (GΩ)      | 1.20 ± 0.14       | 1.37 ± 0.12  | n.s. |
| I_ap (pA)         | 21.53 ± 2.89      | 30.34 ± 3.82 | n.s. |

AP Characteristics

| MEOX2+/+ (N = 26) | MEOX2+/– (N = 43) | P (t-test) |
|-------------------|-------------------|-----------|
| V_memb (mV)       | –49.63 ± 1.06     | –51.03 ± 0.91 | n.s. |
| OS (mV)           | 69.02 ± 0.96      | 66.61 ± 1.50  | n.s. |
| US (mV)           | –66.15 ± 0.84     | –66.30 ± 0.65 | n.s. |
| AP Tresh (mV)     | –30.94 ± 0.93     | –29.90 ± 0.48 | n.s. |
| dV/dt_max (mV·ms⁻¹) | 165.23 ± 16.09    | 153.89 ± 9.09  | n.s. |
| dV/dt_min (mV·ms⁻¹) | –35.15 ± 3.05      | –37.32 ± 1.96  | n.s. |
| t1-t2 (ms)        | 1.51 ± 0.11       | 1.43 ± 0.07   | n.s. |
| t1-t3 (ms)        | 5.88 ± 0.38       | 5.34 ± 0.27   | n.s. |
| t2-t3 (ms)        | 4.37 ± 0.27       | 3.90 ± 0.22   | n.s. |

Afterdepolarization Characteristics

| MEOX2+/+ (N = 26) | MEOX2+/– (N = 43) | P (t-test) |
|-------------------|-------------------|-----------|
| A0 (mV)           | –50.27 ± 1.00     | –51.4 ± 0.84  | n.s. |
| A1                | –25.28 ± 1.35     | –23.80 ± 1.19 | n.s. |
| t1 (ms)           | 97.77 ± 10.61     | 109.05 ± 9.43 | n.s. |
responded with a heat-activated current. The distribution of heat-responsive sensory neurons was similar in Meox2+/−/C0 and littermate control neurons (Fig. 5A). However, the number of action potentials evoked by slow depolarisation was reduced in Meox2+/− sensory neurons compared to controls (Fig. 5B). Ramp-shaped
depolarisation to 1-, 2- and 3- times the individual IAP, revealed a significantly reduced number of action potentials in Meox2+/− neurons (Fig. 5C). Additionally, prolonged membrane depolarisation (20 s) also showed a significantly decreased number of generated action potentials in Meox2+/− sensory neurons (Fig. 5D,E).

We next sought to determine the molecular mechanisms underlying nociceptive deficiencies noted in Meox2+/− animals. Considering that MEOX2 is a transcription factor, we reasoned that there is a dysregulation of genes maintaining nociceptor cellular identity or those regulating pain responses in the DRG. We therefore performed RT-qPCR on total RNA isolated from DRG dissected from 8- to 10-week old sex-matched Meox2+/− and control littermates using primers for genes encoding known markers of various subtypes of nociceptors, Cacna, Ntrk1 and the capsaicin receptor, Trpvl. We did not observe any difference in these genes between DRG harvested from Meox2+/− animals or littermate controls (Fig. 6A). Moreover, we did not observe any difference in expression of Ntrk2 or Ntrk3, markers of mechanoreceptors and proprioceptors respectively, indicating that there was no deficit in the other major subgroups of haploinsufficient MEOX2 sensory neurons (Fig. 6B). We hypothesised that decreased number of APs upon current injection might be a consequence of impaired AP initiation or change in expression of pace-making channels. Since MEOX2 is ubiquitously expressed including excitable tissues such as the central nervous system (Fig. 1B), muscles and heart [28–30], we posited that a nociceptor-specific effect of MEOX2 haploinsufficiency might originate from altered expression of nociceptor-specific ion channels. Therefore, among numerous channels affecting action potential initiation and frequency in DRG neurons, we focused on voltage-gated sodium channels Na1.7 (Scn9a), Na1.8 (Scn10a) and Na1.9 (Scn11a) all of which are preferentially expressed by nociceptors [31,32]. While RT-qPCR revealed no difference in expression levels of Scn10a mRNA between Meox2+/− and Meox2+/+ DRG, two candidates with specific importance in nociceptors, Scn9a and Scn11a mRNA, were significantly downregulated in Meox2+/− (Fig. 6C).

To identify genes dysregulated in MEOX2 deficiency that additionally may modulate the reduction in painful responses to noxious stimuli we performed RNA-seq on DRG dissected from either adult Meox2+/− or control littermate mice. RNAseq results are summarised in a volcano plot of log2 fold change vs. −log 10 (q-value), where differentially expressed genes (DEGs) were considered significantly changed if they had a q-value smaller than 0.1 (Fig. 6D). Levels of Meox2+/− mRNA were 0.3-fold that of controls (q value 0.002) validating our assay and analysis. As a validation of the transcriptome DEGs, we measured the amount of Actn3 mRNA levels by qPCR finding an increase in Actn3 levels of 2.26-fold in Meox2+/− samples (Fig. 6E) while transcriptome data show a 5.4-fold increase in Meox2+/− DRG compared to the controls. Additionally, nine genes were downregulated and 17 genes upregulated ≥ 4 fold of controls (Table 1). Gene ontology (GO) enrichment analysis for cellular process using Gene Ontology enrichment analysis and visualisation tool (GOri, http://ebi-go.technion.ac.il/) revealed that the largest portion of upregulated genes were associated with terms related to function of the muscles like ‘muscle contraction’, ‘sarcomere organisation’ and ‘muscle fibre development’ (Fig. 6F). Downregulated in Meox2+/− DRGs were those genes associated with GO terms ‘extracellular matrix organisation’ and ‘biological adhesion’ as well as ‘animal organ morphogenesis’ (Fig. 6F). Intriguingly, a large portion of these gene products participate in development, cellular specification, pain perception modulation and/or sensory neuronal function. The top downregulated gene was BCL9L, β-catenin transcriptional co-activator with BCL9, whose mRNA abundance showed a 29.9-fold downregulation compared to control values (q-value 0.002). BCL9/BCL9L transcriptional activation by β-catenin is critical in sensory neurogenesis, proliferation and fate specification [33–36]. Hoxc11 (9.8 fold downregulation, q-value 0.039), Hoxd10 and Hoxd11 (5.9-fold downregulation, q-value 0.023) are expressed in the posterior neural tube, dorsal root ganglion and hind limbs, are essential for spinal cord patterning and sensory nervous system [37–39], and were significantly downregulated in Meox2+/− adult DRG. Intriguingly, we uncovered a number of DEGs in Meox2+/− DRG which are already associated with pain perception or peripheral nerve injury, such as Uts2b [40], Mybpc1 and Mtbpc2 [41, 42], Npy [43] and Penk [47].

Discussion

Here we report that homeobox transcription factor MEOX2 is expressed in the DRG with specific markers of a subtype of sensory neurons involved in nociception. MEOX2 haploinsufficiency led to acute and inflammatory pain response deficiency in Meox2+/− murine model. While we noted a significant reduction in behavioural responses to nociceptive challenges such as noxious heat and capsaicin injections, no corresponding deficiencies in Ca2+ influx upon pharmacological stimulations with capsaicin, nor a decrease in
Fig. 6. Mesenchyme homeobox protein 2 (MEOX2) deficiency in the dorsal root ganglia (DRG) causes deregulation of a number of known pain modulator and sensory neuron developmental genes. (A) Levels of mRNA in Meox2−/− (red bars) DRG for nociceptor markers Calca, Ntrk1 and TrpV1 compared to Meox2+/+ littermate DRG controls (black bars) relative to Gapdh. Levels of mRNA for all RT-qPCR experiments are normalised to an average of Meox2+/+ samples. (B) Levels of Ntrk2 and Ntrk3 mRNA in Meox2−/− (red bars) compared to Meox2+/+ littermate DRG controls (black bars) relative to Gapdh. (C) Levels of mRNA in Meox2−/− (red bars) DRG for nociceptor-specific voltage-gated sodium channels Scn9a, Scn10a and Scn11a compared to Meox2+/+ littermate DRG controls (black bars) normalised to Gapdh. (D) Volcano plot of differentially regulated genes depicts significantly up- and down-regulated genes in pooled DRG isolated from three adult Meox2−/− animals as compared to their control littermates, n = 3. X-axis represents log2 fold change and the y-axis represents –log10 (q-values). Genes with q-value (FDR) of less than 0.1 were assigned differentially regulated, and coloured in blue. Top pain- or itch-related genes are labelled. (E) Levels of Actn3 mRNA in Meox2−/− (red bars) compared to Meox2+/+ littermate DRG controls (black bars) normalised to Gapdh, as one of the top upregulated hits in the transcriptome of Meox2−/− DRG. For all RT-qPCR experiments three littermate animals per genotype were used; mean ± SEM; Unpaired Student’s t-test, P-values *< 0.05, or as indicated. (F) The top up- and down-regulated gene ontology (GO) terms for cellular process. Enriched GO terms were analysed using GOrilla, and those with a P-value < 0.001 were considered significant. Top eight GO terms are plotted both the upregulated (right side, marked in red) and downregulated (left side, in blue) with bars representing –log10(P-value) for particular GO term.
capsaicin receptor, TRPV1, mRNA expression were detected. However, we did find decreased expression of Scn9a and Scn11a mRNA in Meox2+/− DRG, and these are likely to be causally involved in the decreased number of action potentials fired upon ramp current injection, thus providing a mechanistic explanation of the nociceptive behavioural phenotype.

The highly conserved family of homeobox genes were originally discovered to regulate tissue patterning in Drosophila [48]. Subsequently, they also participate in early embryonic tissue patterning in vertebrates and maintenance of important functions in adults [49]. In the nervous system, Hox genes are best known for encoding cellular and positional identity along the rostro-caudal axis [50]. Large number of Hox genes are specifically expressed in the DRG [51] and Hoxd11 and Hoxb8 play a role in pain perception. However, loss of Hoxb8 does not affect nociceptor function directly, but alters spinal cord patterning in laminae I and II [24,25]. Conversely, in Meox2+/− we did not detect significant alteration of the IB4 and PKCγ positive laminae of the spinal cord as reported for the loss of Hoxb8 [24]. Nevertheless, MEOX2 is expressed throughout the spinal cord and central nervous system. Therefore, we cannot rule out possible subtle alteration of the dorsal horn patterning we have not detected by immunofluorescence, or the possible unknown contribution of CNS-expressed MEOX2 to pain processing.

Our data demonstrate that MEOX2 is primarily expressed in nuclei of both peptidergic (CGRP+) and non-peptidergic (Na+,1.8+ and IB4+) sensory neurons as revealed by co-labelling immunofluorescence studies of adult mouse DRG sections. Nociceptive responses to temperature, capsaicin and formalin, chemical and inflammatory noxious stimuli respectively, were impaired, suggesting a critical role of MEOX2 in pain responses in mice. We did not detect a reduction of nociceptor marker transcripts including Calca, Scn11a and Ntrk1 or TrpV1 suggesting that overall nociceptor transcriptional identity was not perturbed with MEOX2 haploinsufficiency. This notion is supported by normal PGP9.5 immunoreactivity in the epidermis of the hind paw skin sections from Meox2+/− mice, together suggesting normal abundance and morphology of nociceptors. Additionally, there was no difference in the percentage of heat responsive vs. unresponsive cells in DRG cultures. Nevertheless, impaired behavioural responses to acute noxious stimuli and increased response of cultured DRG cells to MrgprA3 agonist chloroquine and TRPA1 agonist PF-4840154, with no expression level change for Scn9a or Scn11a mRNA in Meox2+/− DRG, and these are likely to be causally involved in the decreased number of action potentials fired upon ramp current injection, thus providing a mechanistic explanation of the nociceptive behavioural phenotype.

### Table 1. Transcriptome analysis of Meox2+/− compared to Meox2+/+ DRGs with top hits (q-value < 0.1, log₂ fold change > 2)

| Upregulated gene | q-value | log₂ fold change | Downregulated gene | q-value | log₂ fold change |
|------------------|---------|------------------|--------------------|---------|------------------|
| Bcatl           | 0.002326| −4.94104         | Ighm1              | 0.002326| 5.97697         |
| Uts2b           | 0.002326| −4.52092         | Lonrf3             | 0.002326| 5.17544         |
| Scr5a2          | 0.002326| −4.0028          | Myh4               | 0.002326| 3.03753         |
| Myh1            | 0.002326| −3.67681         | Mybpc2             | 0.028767| 2.97938         |
| Mybpc1          | 0.010192| −3.42159         | Mylk2              | 0.002326| 2.73385         |
| Hoxc11          | 0.038627| −3.30646         | Mylk4              | 0.004157| 2.60787         |
| Myh2            | 0.00881 | −3.00267         | 1110002E22Rik       | 0.0127  | 2.5692          |
| Hoxd10          | 0.023293| −2.58451         | Actn3              | 0.002326| 2.43554         |
| Sppr1           | 0.002326| −2.32377         | Crmy5              | 0.002326| 2.29361         |
| Fad2            | 0.011493| 2.26576          | Ryr1               | 0.002326| 2.26065         |
| Ckm              | 0.0127  | 2.23488          | Slpi                | 0.002326| 2.19309         |
| Myplf           | 0.002326| 2.14749          | Ampd1              | 0.041605| 2.12606         |
| Myaz1           | 0.020283| 2.04978          | Tmod4              | 0.048909| 2.01775         |
density \( (G_{\text{max}}) \), activation voltage or slope of activation. In spite of slightly increased response to TEA in \( \text{Ca}^{2+} \) imaging, the maintained shape of action potential between \( \text{Meox2}^{-/-} \) and control littermate cells implies that potassium channels involved in setting the resting membrane potential function properly. The chosen high KCl concentrations substantially depolarised cells and activated most voltage-gated calcium channels; the similar responses of \( \text{Meox2}^{-/-} \) and \( \text{Meox2}^{+/+} \) did not indicate major functional changes in these channels. These results imply that basic transducer functions and neuronal excitability were unaffected. Nevertheless, MEOX2 deficiency resulted in a dramatic reduction of action potential firing, more specifically the number of action potentials generated in response to depolarising voltage stimuli. A decrease in specific voltage-gated sodium channel expression, critical in proper AP generation, could be reflected by the impaired number of action potential firing and nociceptor-specific voltage-gated sodium channels \( \text{Na}_1.7 \) and \( \text{Na}_1.9 \). These have been previously described to be threshold channels, having a major role in amplifying subthreshold stimuli and therefore action potential initiation in DRG [57–59]. Importantly, we found that \( \text{Scn}9a \) and \( \text{Scn}11a \) mRNA expression significantly decreased, providing plausible mechanistic evidence that reduced action potential firing in \( \text{Meox2}^{-/-} \) nociceptors, and therefore impaired nociception, stems from decreased expression of \( \text{Na}_1.7 \) and \( \text{Na}_1.9 \). Additionally, reduction of \( \text{Na}_1.7 \) and \( \text{Na}_1.9 \) expression also may explain the reduced latency of response to the hot plate heat challenge. Moreover, our observation is in line with previous reports finding that loss of function \( \text{Scn9a} \) leading to CIP in humans results in decreased action potential firing in patient iPSC-derived nociceptors, especially upon ramp-shaped depolarisation [60]. Additionally, mounting evidence involves \( \text{Na}_1.9 \) in complex physiology of inflammatory pain through histamine [61] as well as other inflammatory mediators induced mechanisms. This provides additional mechanistic evidence that impairment of the second phase of the formalin test in \( \text{Meox2}^{-/-} \) behavioural assays theoretically could originate from decreased sensitivity to inflammatory pain due to downregulated expression of \( \text{Na}_1.9 \). RNAseq analysis also revealed downregulation in several nociceptive-modulating genes including neuropeptides \( \text{Uts}2b, \text{Npy} \) and \( \text{Penk} \) involved in pain signalling and nerve injury [40,44–46]. MEOX2-mediated regulation of a subset of neuropeptides involved in nociception raises novel questions about additional layers of MEOX2-guided functionality of nociceptors. These data raise an interesting possibility of the involvement of MEOX2 in the regulation of sodium channel function in other excitable tissues, especially in the conductive system of the heart.

Transcriptome analysis revealed a number of DEGs speculatively contributing to the nociceptor dysfunction. MEOX2 has been extensively studied for its role in early embryonic patterning, particularly in mesodermic tissue [2,3]. Correspondingly, \( \text{Meox2}^{+/-} \) animals lack specific limb muscles, and a significant reduction of others [4]. Hind limb development is particularly vulnerable to \( \text{Meox2} \) ablation. Indeed, the most significant GO term associated with upregulated genes in \( \text{Meox2}^{+/-} \) DRG transcriptome was ‘muscle system process’, and significantly dysregulated genes include those associated with muscle function such as, \( \text{Actm3}, \text{Myom2}, \text{Ryr1}, \text{Tnn2}, \text{Mybpc1}, \text{Mettl20}, \text{Mylk2}, \text{Ampd1}, \text{Myh1} \) and \( \text{Myh2} \). While we did not detect changes in \( \text{Ca}^{2+} \) influx upon general KCl stimulation, transcriptome analysis did reveal changes in the expression of some voltage-gated calcium channels. In humans, mutations in \( \text{CACNA1S} \), a gene upregulated in mouse \( \text{Meox2}^{+/-} \) DRG encoding the L-type voltage dependent calcium channel, cause types of rare, autosomally inherited periodic paralysis, hypokalemic periodic paralysis type 1 (MIM no. 170400) and susceptibility to thyrotoxic periodic paralysis type 1 (MIM no. 188580). Likewise, \( \text{Ryr1} \), gene encoding a sarcoplasmic reticulum calcium channel in humans, and upregulated in \( \text{Meox2}^{+/-} \) mouse DRG, causes several types of rare autosomal recessive or dominantly inherited neuromuscular or myopathy disorders [62], suggesting that MEOX2 transcriptional activity could be a common mechanism amongst these seemingly disparate neuro/muscular disorders. Of note, \( \text{Meox2}^{-/-} \) mice in this study showed no apparent locomotion defect, nor gait, balance or stamina dysfunctions as analysed by the accelerating Rotarod performance, ruling out the possibility of confounding effects of musculature development and function in pain behaviour interpretation.

We originally observed a significant change in expression of \( \text{MEOX2} \) mRNA in fibroblasts harvested from CIP patients with mutations in a methyl transferase, \( \text{PRDM12} \) [8]. While we found co-labelling of \( \text{PRDM12} \) and MEOX2 in a small number of cells in the adult mouse DRG (not shown), we did not detect dysregulated \( \text{Meox2} \) mRNA levels in classical \( \text{PRDM12} \) knock-out mice [63] nor in specific \( \text{PRDM12} \) DRG-specific knock outs (\( \text{Prdm12}^{fl/fl}; \text{Avil-Cre}^{+/-} \)) by RNAseq [64]. Likewise, we were also unable to detect any changes in \( \text{MEOX2} \) protein levels in \( \text{Prdm12}^{fl/fl}; \text{Avil-Cre}^{+/-} \) DRG by Western blotting (data not shown). A possible explanation for this discrepancy between human fibroblast and mouse DRG could...
be species or tissue differences. It is also possible that PRDM12 and MEOX2 coexpression is much more abundant early in development when PRDM12 expression is less restricted [65]. Therefore, the upstream transcriptional regulation of MEOX2 remains to be elucidated.

We conclude that MEOX2 has a previously undescribed but critically important role in the function of nervous system, by modulating nociception through Na,1.7 and Na,1.9 expression and consequential ability of action potential firing. Whether MEOX2 plays a similar role in other excitable tissues, such as the heart, or in the transition from acute to chronic pain is of great interest in determining the potential of MEOX2 possible therapeutic target.

Materials and methods

Animals

Both male and female mouse littersmates 8–12 weeks of age on C57BL/6J background were used for most assays, with the exception of the intraplantar formalin injection assay, where only male animals were tested due to availability. Animals were housed at Institute of Molecular Biotechnology (IMBA), Vienna, Austria. The mouse facility maintained a 12-h light/dark cycle and provided with food and water ad libitum. Experiments described in this study were approved by the Bundesministerium fur Wissenschaft, Forschung und Wirtschaft (BMWFFW-66.015/0011-WF/V/3b/2017) and carried out according to EU-directive 2010/63/EU. Genotyping was determined using primers previously published: morecre: 5'-GCT GAT TTC TGA GGA TCT G-3' and morerev: 5'-CTA CCC CGA ACT CTC CAC ATC TT-3' [5].

Immunohistochemistry of DRG and spinal cord sections

Lumbar sections of spinal cord or DRG from control or Meox2−/− littersmates were dissected into ice-cold PBS, fixed in 4% PFA for 20 min and washed with PBS. Following cryopreservation in 30% sucrose, 20-µm sections were cut on a cryostat (HM560, Microm International GmbH, Walldorf, Germany) at the Histopathology Service Facility at the Vienna Biocenter Core Facilities (VBCF), member of the Vienna Biocenter (VBC), Austria, mounted on glass slides and stored at −80 °C until needed.

Mounted DRG sections were permeabilised with 0.5% Triton-X100 in PBS for 15 min at room temperature and incubated in Blocking buffer consisting of 10% normal goat serum, 1% BSA in 0.25% Triton-X100 in PBS for 1 h at room temperature to eliminate non-specific binding. Sections were then incubated with primary antibody at 4 °C overnight diluted in Blocking buffer raised against: MEOX2 1 : 50 (LSBio; LS-B5472), CGRP 1 : 500 (Millipore, PC205L, Burlington, MA, USA), Na,1.8 1 : 1000 (Abcam; ab63331), TrkA 1 : 1000 (Abcam; ab76291), or isolectin GS-IB4 1 : 1000 (Thermo Fisher Scientific; I21412, Waltham, MA, USA). Specifically for MEOX2 staining: following blocking, sections were washed briefly with 0.1% Tween-20 in PBS, then incubated in goat F(ab) anti-mouse IgG 1 : 2000 (Abcam; ab6668) diluted in 0.1% Tween 20 in PBS for 1 h and subsequently with primary anti MEOX2 antibody (1 : 50, LSBio;LS-B-5472) overnight. The following day, sections were washed with Washing buffer (0.01% Triton-X100 in PBS) and incubated in appropriate secondary antibodies (Thermo Fisher Scientific) 1 : 500 dilution, nuclear stain DAPI at 0.2 µg·mL−1 (Roth, 6335.1) and where appropriate, IB4 antibody 1 : 1000 (Life Technologies; I21412, Carlsbad, CA, USA), diluted in Washing buffer for 1 h at room temperature. Finally, they were washed in PBS and coverslipped with a drop of Pro-Long Gold antifade mounting media (Invitrogen, P36934, Waltham, MA, USA).

The DRG incubated with anti-CGRP, TrkA, or IB4 together with anti-MEOX2 antibodies were imaged with Slide Scanner Vectra Polaris (Akoya Biosciences) at the Core Facility Imaging, Medical University Vienna. Analysis of the cells were performed with HALO (Indica Labs) with the Multiplex IHC Module to determine the single and double positive cells within the DRG slice. The programme was utilised for cell identification by segmenting the nuclei as DAPI positive structures, and cytoplasm detection using the preset mask. Cells immunopositive for CGRP, TrkA or IB4 alone or co-expressed with MEOX2 were counted by experimenter blinded to the genotypes. Images of DRG sections incubated with anti-Nav1.8 antibody were acquired using the Pannoramic FLASH 250 II slidescanner in 3D Mode at the Histopathology Service Facility at the Vienna Biocenter Core Facilities (VBCF), member of the Vienna Biocenter (VBC), Austria. The individual tissue sections were marked and exported via CaseViewer, the split images were then reassembled to stacks via a Fiji-Macro and the slices with highest contrast were saved for further 2D-analysis. For detecting the cells, a deep learning approach was utilised, performing annotation, training and final segmentation. Annotation was done using Qupath (https://qupath.github.io/) outlining the cells on a subset of images, then the training of the deep learning model was executed in StarDist (https://github.com/stardist/stardist). The final analysis was performed via a custom Fiji-Macro and StarDist, applying the trained model on DRG section images, identifying the nuclei, expanding to the cytoplasm, and eliminating significant overlaps from further analysis. To define the outlines of the ganglion itself, the cell bodies were merged using a closing operation and holes were filled. The cell regions obtained via StarDist were used to quantify the signal in red (Nav1.8), green (Meox2) and blue...
Western blotting

After sacrificing animals, tissue samples were collected in RIPA (Sigma-Aldrich, St. Louis, MO, USA) buffer on ice, with Proteinase and Phosphatase Inhibitors Cocktail (Thermo Fischer Scientific), lysed and homogenised using a Precellys 24 Tissue Homogenizer (Bertin Instruments, 3 × 30 s, 5000 r.p.m.) and protein concentration measured using the Bradford assay (Bio-Rad). Laemmli buffer (Sigma) treated samples with standardised concentration of 1 µg·µL−1 were incubated at 95 ºC for 5 min. Per lane 25 µg of protein was loaded on 10% PAA gel, followed by protein transfer to a methanol activated PVDF membrane (GE Healthcare Life Sciences) over night at 4 ºC with constant current of 0.12 A. The membrane was blocked with 5% BSA in TBS-T (0.01% Tween 20) for 1 h at room temperature. Anti-MEOX2 1 : 1000 (LS Bio; LS-B5472) and GAPDH 1 : 1000 (HRP conjugate, Cell signalling; 3683S, Danvers, MA, USA) or HSP90 1 : 1000 (Cell signalling, C45G5) antibodies diluted in Blocking buffer were used for detection of MEOX2 or loading controls respectively, followed by anti-rabbit or anti-mouse HRP-conjugated secondary antibodies 1 : 30 000 (Cytiva, NA934V; Sigma-Aldrich, GENA931), if required, also diluted in Blocking buffer. The signal was visualised with a chemiluminescent reagent (ECL, GE Healthcare Life Sciences) and imaged (ChemiDoc Imaging System, Bio-Rad).

Behavioural assays

All pain assays were performed once on each animal, separated by at least 24 h between tests to reduce stress.

Hot plate assay

Briefly, the first day of testing, animals were placed on a hot plate (Ugo Basile) set for 50 ºC and manually observed for first reaction. Counted reactions included: jumping, licking, shaking or lifting of the hind paws. The animal was removed from the apparatus at first reaction or when no reaction was observed within 60 s. 24 h later, animals were tested for first reaction to being placed on a 52 ºC hot plate.

Intraplantar capsaicin or formalin injections

1 µg of capsaicin (Sigma; M2028) diluted in 15 µL PBS was injected intraplantar in the hind paw. The animal was then observed for 5 min and timed for duration of reaction. 2.5% formalin (830 mM, Thermo Fischer Scientific) was diluted in 20 µL PBS and was injected in the hind paw of the experimental animal which was then observed for 50 min and timed for the duration of reaction. Counted reactions for both tests included: licking, shaking or lifting of the injected paw. In response to the formalin test,
reaction time per 2 min bins was reported. At the completion of the test, the animal was returned to its home cage.

**Accelerating rotarod performance test**

Accelerating rotarod was performed on a 5–40 r.p.m. accelerating apparatus (Ugo Basile), as described previously [14]. Briefly, mice were habituated to the rotating rotarod apparatus (Ugo Basile) once for a 1-min trial, at 5 r.p.m. Following habituation, each mouse was given one trial per day, for four consecutive days, on the 5–40 r.p.m. accelerating rod.

**Sensory neuron culture**

Lumbar DRG were harvested from adult mice as previously published [15,16]. After removal of the connective tissue from the ganglia, they were incubated twice in Liberase (90 mg·L⁻¹ DMEM) for 30 min. After washing with PBS, the tissue was incubated with 0.05% Trypsin-EDTA for 15 min and subsequently washed with TNB medium (Biochrom, Merck Millipore). The sensory neurons were dissociated with a fire-polished Pasteur pipette and centrifuged through a 3.5% BSA gradient to eliminate debris and non-neuronal cells. The pelleted sensory neurons were resuspended, plated on coverslips coated with poly-t-l-lysine/laminin-1 (Sigma-Aldrich), and cultivated in supplemented TNB containing mNGF 2.5 s (25 ng·mL⁻¹) and protein-lipid complex (Biochrom, Merck Millipore). The DRG were dissociated with a fire-polished Pasteur pipette and centrifuged through a 3.5% BSA gradient to eliminate debris and non-neuronal cells. The pelleted sensory neurons were resuspended, plated on coverslips coated with poly-t-l-lysine/laminin-1 (Sigma-Aldrich), and cultivated in supplemented TNB containing mNGF 2.5 s (25 ng·mL⁻¹) and 5% CO₂ in a humidified incubator.

**Single cell electrophysiology**

Cultured sensory neurons were used for electrophysiological experiments 16–24 h after seeding. Glass coverslips were mounted in a recording chamber and placed on a Zeiss Axiovert 200 microscope. All measurements were recorded with an EPC 10 and the PATCHMASTER v2.73 software (HEKA) at room temperature. From isolated sensory neurons, membrane potential was recorded in single-cell current-clamp configuration of the patch clamp technique in extracellular solution (ECS) containing (in mmol·L⁻¹): NaCl (150), KCl (5), CaCl₂ (2), MgCl₂ (1), HEPES (10), Glucose (10) and the pH was set to 7.3 with NaOH. Borosilicate glass pipettes (Science Products) were pulled with a horizontal puller (P-97, Sutter Instruments Company) and filled with an intracellular solution composed of (in mmol·L⁻¹): K gluconate (98), KCl (50), CaCl₂ (0.5), MgCl₂ (2), EGTA (5), HEPES (10), MgATP (2), NaGTP (0.2) and pH adjusted to 7.3 with KOH. The recorded neurons were held at 0 pA. The minimal current to evoke a single action potential within 50 ms (IAP) was obtained by 5 pA increasing depolarising pulses. For the slow depolarisation (5 s) of the membrane, current injections where applied from 0 pA to 1×, 2× and 3× IAP of the respective neuron and amplitude of the 20 s depolarising pulse was set to 2× IAP [16].

A seven-barrel application system (Dittel, Prague) with common outlet was used for heat stimulation of single neurons [17]. Heat-activated inward currents (IHeat) were elicited by applying ramp-shaped heat stimuli (linear temperature increase from room temperature to 50 °C within 5 s).

The input resistance of sensory neurons was determined by four-increasing hyperpolarising current injection steps (Δ = 5 pA per step from a holding current of 0 pA, 5 kHz). The average resistance was calculated according to Ohm’s law. From the AP evoked by injecting depolarising current pulses (50 ms, sampled at 20 kHz), the resting membrane potential (Vmem), afterhyperpolarisation and overshoot (OS) as well as the rheobase in Meox₂⁺/− and Meox₂⁻/− DRG neurons were determined. From the first derivative of the evoked APs, the maximal speed of depolarisation and the biphasic repolarisation were derived. The AP threshold was determined as the turning point of the first derivative and the according membrane voltage was deducted [15,16,18].

**Microfluorimetric Ca²⁺ measurements**

Sensory neuron cultures for calcium imaging were prepared similarly to the method described above, with the difference that DRG from all spinal levels were used, from two Meox₂⁺/− and two Meox₂⁻/− adult 18 weeks old male mice. DRG were digested in Dulbecco’s modified Eagle’s medium (DMEM) containing 1% streptomycin/penicillin, treated with 1 mg·mL⁻¹ collagenase (Sigma-Aldrich) and 3 mg·mL⁻¹ Dispase II (Roche Diagnostics) for 60 min, mechanically dissociated with a Pasteur pipette and plated onto 12 mm glass coverslips previously coated with 100 µg·mL⁻¹ poly-D-lysine (Sigma-Aldrich). DRG neurons were cultured in DMEM culture medium supplemented with 10% BSA and 100 µg·mL⁻¹ streptomycin/penicillin (all from Biochrom, Berlin, Germany) 2% B-27 Supplement (50×, serum free, Gibco; 17504044). Neurons were cultured at 37 °C and 5% CO₂ for 15–30 h and loaded with Fura-2 AM (3 µM, 30 min at 37 °C, containing also 0.02% Pluronic, both from Life Technologies GmbH, Darmstadt, Germany) and left to recover for about 10 min in ECS (as above except for a lower calcium concentration of 1.25 mmol·L⁻¹ CaCl₂), which was also used as continuous gravity-driven superfusion in the experiments. The coverslips were placed in glass bottom dishes which were then mounted in an Olympus IX73 inverted microscope and imaged using a 10x objective. Time-course experiments were done using a software-controlled eight-channel common-outlet system (ALA Scientific Instruments Inc., Farmingdale, New York, USA) with cells permanently superfused with ECS or stimuli dissolved in ECS, as
follows: chloroquine 100 μM (Sigma-Aldrich), WS12 500 nm (Santa Cruz Biotechnologies Inc., Dallas, TX, USA), PF-4840154 1 μM (MedChemExpress), capsaicin 1 μM (Sigma-Aldrich), TEA 3 mM (Sigma-Aldrich). Stimulation with an ECS containing KCl 60 mM (isosmolar substitution of NaCl) served as a positive control. Cells were superfused with stimuli solutions for 30 s, followed by 270 s superfusion with ECS. Fura-2 was alternately excited for 30 ms by a 340 nm (50 mWatt, used at 100%) and 30 ms by a 385 nm (1435 mWatt, used at 5%) LED using an Omicron LEDHub (Omicron-Laserage Laserprodukte, Rodgau-Dudenhofen, Germany). Fluorescence emission was long-pass filtered at 495 nm and pairs of images were acquired at a rate of 1 Hz with a 4.2 Megapixel 16 bit CCD camera (6.5 μm pixel edge length, 18.8 mm sensor diameter, PRIME BSI, Teledyne Photometrics, Tucson, Arizona USA). The hardware was controlled by the μmanager 1.4 plugin in IMAGEJ. The background intensity was subtracted before calculating the ratio between the fluorescence emitted when the dye was excited at 340 nm and at 385 nm (F340/F385 nm). The time course of this ratio was analysed for regions of interest adapted to individual cells. The area under the curve of the stimulation periods was analysed for regions of interest adapted to individual cells. The area under the curve of the stimulation periods was used to quantify response magnitude. The calculation uses a reference period immediately before the period of interest, the resulting unit of Ratio*time was omitted for simplicity.

RNA sequencing

Dorsal root ganglia were isolated from 3 Meox2%-/- and 3 Meox2%-/+ littermates (14 ± 2 weeks of age) into RNAlater™ Stabilisation Solution (Thermo Fisher) and frozen at −80 °C. Tissues were homogenised in buffer RLT using a TissueLyser II (Qiagen, Hilden, Germany 10 × 30 s, 30 Hz) before centrifugation through QIAshredder spin columns (Qiagen). RNA was isolated using an RNEasy Mini Kit (Qiagen), following the manufacturer’s instructions. The amount of RNA was quantified using a Qubit 2.0 Fluorometric Quantitation system (Life Technologies) and an Experion Automated Electrophoresis System (Bio-Rad) was used to calculate RNA integrity score. RNA-seq libraries were prepared with the TruSeq Stranded mRNA LT sample preparation kit (Illumina) using Scilone and Zephyr liquid handling robotics (PerkinElmer) at pre- and post-PCR steps. Library concentrations were determined using a Qubit 2.0 Fluorometric Quantitation system (Life Technologies) and the distribution of sizes determined using an Experion Automated Electrophoresis System (Bio-Rad). To sequence, the nine libraries were pooled, diluted to equimolar amounts and sequenced on an Illumina HiSeq 3000/4000 using 50 bp single-end chemistry. Base calls provided by the real-time analysis software (Illumina), were converted into multiplexed, unaligned BAM format before demultiplexing into sample-specific unaligned BAM files. Custom programs based on Picard tools (https://broadinstitute.github.io/picard/) were used for raw data processing.

Transcriptome analysis

Transcriptome analysis was performed with the Tuxedo suite. Per sample, NGS samples (passing vendor quality filtering) were aligned to the UCSC Genome Browser (hg38/mm10) flavour of the Genome Reference Consortium (GRCh38/GRCm38) assembly with TopHat2 (v2.1.1) [19], a splice junction mapper utilising the Bowtie2 (v2.2.9) [20] short read aligner. Basic Ensembl transcript annotation from version e87 (December 2016) served as a reference transcriptome. Cufflinks (v2.2.1) [21] was used for transcriptome assembly, on the basis of spliced read alignments and the reference transcriptome, as well as raw transcript quantification. Transcriptome sets of each sample of each group were combined using the Cuffmerge algorithm, then the differential expression calling was done using Cuffdiff [22]. The cummerbund and biomaRt Bioconductor packages (www.bioconductor.org/packages/release/bioc/html/cummeRbund.html and www.bioconductor.org/packages/release/bioc/html/biomaRt.html) were utilised in custom R

Table 2. List of primers used for RT-qPCR

| Gene  | Protein product | Forward primer (5’→3’ ) | Reverse primer (5’→3’ ) |
|-------|----------------|-------------------------|-------------------------|
| Gapdh | GAPDH          | GTCGTAATGGGGCCTGTCACC   | CACCCCATGACGAACATGGGAGC |
| MEOX2 | MEOX2          | GCTGTATTCTTAGGAGGACATCT | CTACCCGCAGACTCTCCACATTT |
| Cacna1 | CACNA1        | GAGCAGCTTCGAGATGTCAATC | GTGACACTAGAAGCCTCAGAC  |
| Ntrk1 | TrkA           | AACAACGGGAACACTACCCCT   | TGTGCTGTACCGTCACCT     |
| Ntrk2 | TrkB           | GATCTCGGTGGCTGTCGAGAC   | GTGTTCCCTAAAGGACATGT GTA |
| Ntrk3 | TrkC           | TACTCACGGGTGGAGGAACAC   | CCGTGTTGAAGGGGCAGAC   |
| Scn1a | Nav1.1         | TTGGCGGAATTCACCTTCTCTC | CACGATGTGTTTTTATAGCTCAG |
| Scn1a | Nav1.1         | ACCGACAAAGGACGAGAGGAG   | ACAGACTGAAGAGGACGATCA C |
| Scn1a | Nav1.1         | CAATCAGAGCAAGAAAGTTAGG | TGCCCTCTAGATTGAGCAC    |
| Kvna2 | Kv1.2          | ATGGCATATCTGCTGGTCT    | CTACGAGTTAGTGTGCTGA    |
| Kcnn2 | Kv2.1          | CGTACCTGAGCAGCTCTCATG  | CAACCTCAGCTCAGTCAAGCCA |
| Actn3 | ACTN3          | GAGGAGCGACACTAGAGAGGA  | GCCAGTTATTGAAGGGGGCT  |
scripts to perform quality assessment and further refinement of analysis results.

**Real time-qPCR**

The DRG from three *Meox2*+/+ and three *Meox2*+/- mice were isolated into TRIzol Reagent (Thermo Fisher). Tissues were lysed and homogenised using a Precellys 24 Tissue Homogenizer (Bertin Instruments, 3 x 15 s, 5000 r.p.m.) the RNA isolated, treated with PerfeCTa DNase I (Quantabio) and reverse-transcribed using iScript cDNA Synthesis Kit (Bio-Rad), following the manufacturer’s instructions. cDNA amounts were normalised after quantification on a Nanodrop™ 2000 Spectrophotometer. RT-qPCR was performed using an iTaq Universal SYBR Green Supermix (Bio Rad) on a StepOnePlus Real-Time PCR System (Applied Biosystems) with primers as listed in Table 2. The comparative Ct method (ΔΔCT) was used to determine relative gene expression, normalising to *Gapdh*.

**Data-analyses and statistics**

All values are given as mean ± SEM. Data were analysed using ORIGIN software (Originlab) for electrophysiological experiments. Statistical analysis was performed in GRAPHPAD Prism 7.0 by unpaired Students t-test, one-way or two-way ANOVA and Tukey or Sidak post hoc testing and Chi-square test, where appropriate. Differences were considered statistically different if *P* < 0.05.

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**Conflict of interest**

The authors declare no conflict of interest.

**Author contributions**

TK performed and analysed the calcium imaging and RNAseq experiments and contributed to the data interpretation and manuscript conceptualisation, EML performed immunohistochemical experiments and aided in behavioural experiments, ML performed and analysed the electrophysiological experiments and contributed to the data interpretation and manuscript conceptualisation, CIC and MJMF supervised the calcium imaging experiments, CWF performed and analysed the RT-qPCR experiments, AS performed immunohistochemical experiments, MK supervised the electrophysiological experiments and edited the manuscript draft, JMP contributed to the conceptualisation of the project and provided the mouse model, VN conceptualised, designed, lead, organised and supervised the project, performed and analysed the behavioural data, interpreted the data and wrote the manuscript. All authors contributed to and edited the manuscript.

**Data availability statement**

All data generated or analysed during this study are included in this published article in the form of graphs (under the Supporting information). The raw data used and/or analysed during the current study are available from the corresponding author on reasonable request.

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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Supporting information**