A congenital pain insensitivity mutation in the nerve growth factor gene uncouples nociception from affective pain in heterozygous humans and mice

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Ethics statement on mouse experiments

All animal procedures were approved by the Italian Ministry of Health and were fully compliant with Italian (Ministry of Health guidelines, Legislative Decree n°26/2014) and European Union (Directive n°2010/63/UE) laws on animal research. The experiments were carried out in strict accordance with the approved guidelines. In addition, the principles of the Basel Declaration, including the "3R" concept, have been considered throughout the whole project.

Human NGF^{R100W} purification

Human NGF^{R100W} cDNA was cloned into the prokaryotic expression vector pET19b downstream the sequence of the human BDNF prodomain, to produce a chimeric human proBDNF/NGF^{R100W} construct, and expressed in the *E. coli* strain Rosetta(DE3)PLysS. The corresponding chimeric protein was refolded from inclusion bodies and purified using an adaptation of the protocol used for proNGF in [1]. The purified proBDNF-NGF^{R100W} was proteolitically processed with trypsin to produce mature NGF^{R100W}, as previously described[1].

Dorsal root ganglia neurons primary cultures

Dorsal root ganglia (DRGs) neurons were prepared from neonatal (5 days, P5) Wistar rats (Charles River, I) from both sexes, as reported [2,3]. Briefly, DRGs were collected, incubated for 1 hour at 37°C with 0.125% collagenase (Sigma-Aldrich), mechanically dissociated and plated onto coverslips or Petri dishes pretreated with 10 μg/mL poly-L-lysine (Sigma-Aldrich), at a density of 50000 cells/well of a 48 well tissue culture plate. DRG cultures were maintained in serum-free medium, consisting of Dulbecco's modified Eagle's medium

(DMEM)/F12 (Invitrogen) supplemented with 87.5 ng/mL 5-fluoro-2′-deoxyuridine, 37.5 ng/mL uridine, 50 U/mL penicillin and 50 µg/mL streptomycin (all from Sigma) and 0.05% N2 supplement (Invitrogen) at 37 °C in 5% CO_2. The treatment with N2 supplement allows the presence of a physiological level of growth factors, thus preventing the occurrence of a neurotrophin withdrawal state. After 2–3 days in vitro, DRG cultures were stimulated for experimental procedures using either control human NGF^{WT} or human NGF^{R100W} (100 ng/ml) for 5 days, or maintained in basal medium conditions (CTRL). At the end of this incubation period, B2R and phospho-TRPV1 (pTRPV1) protein expression was measured by Western blot after 3 h bradykinin (1 µM) application (antibodies used: rabbit anti-B2R, 1:1000, Alomone Labs; rabbit anti-pTRPV1 and anti-TRPV1, both 1:1000, Millipore). Substance P (SP) was quantified in the culture medium using commercial enzyme immunoassay (EIA), according to the manufacturer's instructions (Cayman Chemical, MI, USA).

To characterize neuronal viability, DRG cultures were fixed in 4% PFA for 10 min at RT, incubated O/N at 4°C with mouse anti-NeuN (1:200, Sigma-Aldrich), and rabbit anti-Neurofilament 200 (1:200, Sigma-Aldrich) followed by goat anti-mouse secondary antibody (1:400, Sigma-Aldrich), goat anti-rabbit rhodamine-conjugated secondary antibody (1:1000, Sigma-Aldrich) and Hoechst 33258 (0.25 µg/ml) for 1 h and 5 min, respectively, at RT. Fluorescence images were acquired using an Olympus BX51 microscope and a 60× oil immersion objective (N.A. 1.4). The number of NeuN-immunoreactive cells was normalized on the total number of cells (i.e., Hoechst-stained). At least 40 microscopic fields per coverslip, in four coverslips from three independent experiments, were quantified for each experimental group.

Hek293 cells culture

Hek293 cells were maintained at 37°C, 5% CO_2, in DMEM/F-12 medium supplemented with 10% FBS, 1% L-Glutamine and 1% penicillin/streptomycin (Gibco-ThermoFisher). Hek293 cells were transfected with pCMV6-XL5-human NGF^{WT} and pCMV6-XL5-human NGF^{R100W} plasmids following the manufacturer's instructions for Lipofectamine 2000 (Invitrogen). 48h after transfection, the supernatants were immunoprecipitated and subjected to Western blotting as described below (see NGF immunoprecipitation and western blot).

Generation of knock in human $NGF^{h/m}$ and $NGF^{R100W/m}$ mice

pCMV6-XL5-human NGF^{WT} plasmid was obtained from OriGene Company (MD, USA; #SC123827) and pCMV6-XL5-hNGF^{R100W} was generated using site-specific mutagenesis PCR.

The targeting constructs were generated using classical cloning technologies. Briefly, a BAC (clone RP24-160F12) containing the entire regions of interest flaking the NGF sequence, was used to generate intermediate plasmids by cloning in pBluescript SK(-) the 5' homology arm (from 89489 to 94076, restriction site MfeI) and 3' homology arm (from 94803 to 99710, restriction site HindIII). The human NGF coding sequence (OriGene #SC123827) and the human NGF^{R100W}, obtained by PCR, were cloned in pBluescript SK(-). Then, the coding regions (WT and R100W), flanked by the homology arms were cloned in pKO2.1 targeting vector carrying the DTA negative selection cassette (kindly provided by Dr. L. Ronfani). Neo cassette was cloned blunt in AgeI site downstream of hNGF (Suppl. Fig. 1). The final targeting vectors were linearized prior to

electroporation using the NotI restriction site. The targeting vectors were transfected into R1p.15 cells (background SV129) and positive clones were selected using Neomycin resistance.

Southern Blot analysis

Genomic DNA was extracted by means of phenol:chloroform:isoamyl alcohol from about 350 cell clones electroporated with either WT or R100W targeting vectors. DNAs were incubated first with EcoRI (for 5' screening), then positive clones were confirmed with XbaI digestion (for 3' screening). Digestions were run in a 0.8% agarose gel O/N at 50 V. After a mild depurination and denaturation, gels were blotted on nitrocellulose and filters incubated with an external 5' or 3' probe (Suppl. Fig. 1). The 5' probe labels a 10.7 kb EcoRI band in the WT allele (Suppl. Fig. 1A) and a 5.2 kb EcoRI band in recombinant alleles (Suppl. Fig. 1B). The 3' probe labels an 8 kb XbaI band in the WT allele (Suppl. Fig. 1A) and a 12.7 kb XbaI band in recombinant alleles (Suppl. Fig. 1B).

Positive clones were injected into the blastocysts of C57BL/6 mice and chimeric animals were obtained.

Mice were genotyped by PCR. The following PCR primers were used:

fw_human: 5'-TTTAGCACCCAGCCTCCCCGTGAAG-3'

fw_mouse: 5'-CAGAAGGAGACTCTGTCCCTG-3'

rev_human-mouse: 5'-CACCTCCTTGCCCTTGATGTCTG-3'

Band sizes are: wild-type 400 bp, mutant 200 bp (Suppl. Fig. 1C).

Behavioral analyses

Experiments were performed on $NGF^{m/m}$, $NGF^{R100W/m}$, $NGF^{h/m}$, $mNGF^{+/+}$, $mNGF^{+/-}$ mice. Mice were kept under a 12 h / 12 h light/dark cycle, with food and water *ad libitum*.

NGF treatment

Mouse wild type NGF was administered daily at the dose of 1 µg/kg to pregnant dams by subcutaneous injection; treatment was protracted until 10 days after delivery. From postnatal day (P) 10 to P60, pups received a daily subcutaneous injection (1 µg/ kg) and intranasal administration (480 ng/kg) of NGF.

Object recognition test

The apparatus consisted of a PVC arena (60 × 60 × 30 cm) with white floor and black walls. The test was performed in 3 days. On day 1, mice were subjected to a habituation phase in which they received two 5-min sessions in the empty arena, separated by a 30-min interval. On day 2, mice were exposed to two identical objects for 7 min to evaluate the total time of exploration. On day 3, mice were placed back in the arena and exposed to a familiar object and another novel object (memory phase). The time spent exploring each object was recorded.

Morris water maze (MWM)

The test was performed in a water tank (120 cm diameter) filled with white opaque water. The platform, placed in the center of SW quadrant, was submerged 1 cm below the water surface. 6-months-old mice were trained with 2 trials per day, with a 40 min interval, for 9 consecutive days. Mice were allowed up to 2 min to locate the platform and the latency to reach it was recorded. If the mouse failed, the experimenter guided it onto the platform. Data were acquired and analyzed using an automated tracking system (Noldus Ethovision XT, NE).

Y-maze test

A PVC maze consisting of three identical arms (40 × 13 × 10 cm) that converged at an equal angle was employed. Each mouse was placed in the center of the maze and allowed to explore freely the arms during an 8-min session. An entry was scored when the mouse was at least halfway through an arm of the maze. An alternation was scored when all the three arms were entered on consecutive events. The percentage of spontaneous alternations (% SAP) was calculated according to the following formula: % SAP = number of alternations / total entries × 100.

Elevated plus maze

The elevated plus maze consisted of two closed arms and two open arms (each 30 × 5 cm) extending from a central platform at 90°. Mice were placed on the central platform and allowed to freely explore the maze; the times spent in the open arms, closed arms and center were measured[4].

Marble burying test

Mice were put O/N in a cage containing 6 glass marbles placed on a 5-cm deep layer of bedding. The next morning, mice were removed from the cage and the number of marbles buried with bedding at least to 2/3 of their diameter was counted[5].

Nest building test

Following the protocol reported in [6], 1 h before the onset of the dark phase, mice were put in a testing cage containing the material (paper) for a nest. The following morning, the nest was examined and its appearance was classified according to the published scoring scale (1 to 5).

Hot plate test

Mice were placed on a surface heated from 42°C to 54°C with 3°C steps. Animals were sequentially tested, allowing a 10-min resting period between each temperature step. The temperature threshold required to observe paw licking and the time required to observe this reaction at each temperature step were recorded.

Cold sensitivity test

Mice were put in a plastic cage and habituated for 30 min. Acetone (50 µl; Sigma-Aldrich) was sprayed onto the plantar surface of the hind paw using a Gilson pipette and the responses were reported as a four-point score: 0 = no response, 1 = brisk withdrawal or flick of the paw, 2 = repeated flicking of paw, 3 = repeated flicking of the hind paw with licking. Acetone was applied six times, alternating between paws, with an interval of 5 min between each application. The frequency of response, expressed as a percentage (number of trial characterized by a response / total number of trials) was evaluated.

Capsaicin injection test

Mice were placed individually in a Plexiglas box for 15 min before drug injection to allow habituation. Capsaicin (Abcam, UK; #141000) was dissolved in dimethyl sulfoxide (DMSO) and injected in the ventral surface of the right hind paw using a Hamilton syringe at a concentration of 3 µg/µl in saline solution (total injection volume 10 µl; 0.1% DMSO final concentration). Control mice were injected with 10 µl of 0.1% DMSO in saline. Following the injection, mice were observed for 15 min and the amount of time spent licking and/or lifting the injected paw was measured.

Tape response assay

Mice were habituated to a plexiglass container for 5 min and then a 3-cm piece of adhesive tape was applied to the back. Mice were observed for 5 min to measure the latency to the first tape removal attempt and the total number of attempts.

Cotton swab assay

Mice were placed in an arena consisting of an elevated chamber with a grid floor and allowed to habituate for 1 h. A cotton swab was stroked through the floor along the plantar paw surface five times, alternating between paws with a 10-s interval. The number of withdrawals were counted and expressed as percentage of the total number of trials.

***In vivo* nociceptive assay**

As reported in [7], CD1 male mice (Charles River, I) were subjected to a mechanical allodynia behavioral test after the injection of either WT or R100W NGF in the hind paw plantar surface at a 0.2 µg/µl concentration (corresponding to 4 µg in a total injection volume of 20 µl) in saline. Control mice were injected with 20 µl of saline. The von Frey test (Ugo Basile, I) was performed before treatment and 1, 3, 4 and 5 h post-injection.

Three-chamber test

The animal was placed in a rectangular arena (70 × 40 × 30 cm) separated into three equal chambers. The test consisted of a first habituation session in which the mouse was free to explore the three chambers for 10 min. Then, an empty cup and a cup containing a stranger mouse were introduced in the side chambers for a 10 min sociability session, and the time spent exploring both chambers was measured.

Cued fear conditioning

A sample group of adult mice (two- and six-months old) was subjected to a preliminary trial to evaluate the foot-shock electric threshold; the resulting response was scored as: 0 = no response, 1 = flinch, 2 = jump/run, 3 = vocalization. The threshold was set as the minimal current intensity causing a "score 3" response. The test was performed in three days. On day 1, mice were habituated to the apparatus, i.e., were left free to explore the testing chamber for 3 min; on day 2, mice were subjected to the "association phase" characterized by 5 repetitions of a tone lasting 10 s that, at second 9, was associated to a 1-s electrical stimulus. On day 3, mice were put in a different chamber and in the "recall phase", the 1-s tone alone was repeated 8 times and the amount of time spent in freezing was assessed. Freezing was defined as the complete absence of motion,

including motion of the vibrissae, for a minimum of 0.5 s. On day 3, mice were transcardially perfused with 4% PFA in PBS (pH 7.4) within 90 min from the end of the recall phase, and the brains were collected to perform c-Fos immunohistochemistry (see above for method). The number of c-Fos-immunoreactive cells was manually counted using ImageJ software and normalized on the area of interest.

Contextual fear conditioning

On day 1, mice were subjected to a habituation phase, i.e., they were left free to explore the chamber for 3 min; on day 2, mice were placed again in the testing chamber and received 5 electrical stimuli. On day 3, mice were put in the same context and the amount of time spent in freezing time was assessed (recall phase); after 1 h, mice were put in a different context (reversal phase) and the freezing time was measured.

Predator fear

The experiments were performed according to the protocol reported in [8]. Briefly, on days 1 to 3, mice were free to explore the entire apparatus in a single 20-min daily session (habituation phase); on day 4, after 10 min of exploration, mice were confined to the stimulus compartment and exposed for 10 s to a rat (predator phase). The behavior in the first 3 min of days 1-3 and in the first 3 min after rat exposure were scored by quantifying the time spent in immobility or locomotion.

Immunohistochemistry

Mice were transcardially perfused with 4% PFA in PBS (pH 7.4), and brains were dissected and post-fixed O/N in the same solution, then cryoprotected in 30% sucrose in PBS for 36 h. The brains were sectioned with a sliding freezing microtome (Leica) to obtain 45 µm-thick coronal sections that were washed 3 times in TBS with 0.3% Triton X-100, then treated with 3.5% H_20_2 in TBS to inactivate endogenous peroxidases. Sections were blocked for 30 min with 10% FBS, 0.3% Triton X-100 in TBS, followed by an O/N incubation at 4°C with either 1:500 goat anti-ChAT (Millipore #AB144P), 1:1000 rabbit anti-c-Fos (Millipore #ABE457), 1:1000 rabbit anti-oxytocin (Millipore #AB911). Biotinylated secondary antibodies (Vector Labs) were diluted in 10% FBS in PBS for 3 h at RT. Finally, sections were incubated in Vectastain ABC HRP Kit (Vector Labs, CA, USA) in PBS for 1 h, followed by another incubation in TBS solution containing 3,3'-diaminobenzidine HCl (DAB, Sigma-Aldrich) and the enzyme Glucose Oxidase Type VII (Sigma-Aldrich); the reaction was stopped after 10 min. Stained sections were mounted on glass slides using DPX medium.
Images were acquired with a Nikon Eclipse E600 optical microscope and the density of immunoreactive cells was calculated using ImageJ.

Skin and DRG immunofluorescence

DRG from adult mice (two- and six-month old) were collected in an eppendorf tube containing cold PBS, then post-fixed in 4% PFA for 30 min at RT, embedded in 2% agarose and sectioned at 50 µm thickness using a vibratome. Sections were washed twice with PBS-Triton 0.3%, then subjected to a 30-min blocking step in 5% NGS and 0.3% Triton X-100 in PBS, followed by an O/N incubation at 4°C with primary antibodies diluted as shown below. AlexaFluor-conjugated secondary antibodies (Thermo Fisher Scientific, MA, USA) were

diluted 1:1000 in 0.3% Triton X-100, 5% NGS in PBS for 2 h at RT. Sections were mounted using Prolong Gold medium (Invitrogen, CA, USA).

For immunofluorescence analysis, the hairy and glabrous skins were collected, allowed to dry and post-fixed in 4% PFA at 4°C O/N, then incubated in 30% sucrose in PBS and frozen in OCT medium (Leica, Germany). Sections (50-µm thick for hairy skin, 20-µm thick for glabrous skin) were obtained using a cryostat. Immunostaining was performed as described above.

The antibodies and dilutions used were: 1:500 mouse anti-NF200 (Sigma-Aldrich, Germany), 1:200 mouse anti-CGRP (Rockland, PA, USA), 1:100 isolectin GS-B4-biotin conjugate (Invitrogen), 1:200 rabbit anti-B2R (Alomone Labs, IL), 1:300 mouse anti-TRPV1 (Millipore, MA, USA), 1:200 rabbit anti-PGP 9.5 (Dako-Agilent, CA, USA), 1:300 rabbit anti- NGF M20 (Santa Cruz Biotech., TX, USA).

All images were acquired with a Leica SP5 confocal microscope and analyzed with Fiji (NIH, MD, USA).

Electrophysiological recordings

Mice were sacrificed using CO_2 inhalation and the saphenous nerve was dissected and placed in an organ bath[9]. The chamber was perfused with a synthetic interstitial fluid (SIF) buffer containing (in mM): NaCl 123, KCl 3.5, $MgSO_4$ 0.7, NaH_2PO_4 1.7, $CaCl_2$ 2.0, Na-gluconate 9.5, glucose 5.5, sucrose 7.5, and HEPES 10, pH 7.4) at 3 ml/min at 32°C. The distal part of the nerve was placed in the organ bath, while the proximal part was placed in an adjacent chamber filled with mineral oil for recording. An electric probe was used to stimulate the nerve and a compound action potential was recorded and analyzed using LabChart4 (AD Instruments, AU) software. Each electrical stimulus elicited a response consisting of three peaks, corresponding to the Aβ, Aδ and C fibers, respectively. To measure the conduction velocity of each fiber type, the time elapsed from the beginning of the stimulus to the appearance of the peak was divided by the distance between the electric probe and the recording electrode.

NGF immunoprecipitation and western blot

Cerebral cortices were isolated from adult mice and homogenized in lysis buffer (Tris-HCl 100 mM, NaCl 400 mM, SDS 0.1%, Triton X-100 1%). The homogenates were sonicated, incubated in ice for 30 min and centrifuged at 15000×g for 30 min at 4°C. Protein concentration in the supernatant was quantified using the Bradford method (Bio-Rad, CA, USA). 4 mg of protein were immunoprecipitated with an excess of anti-NGF αD11 antibody in NET gel buffer (Tris-HCl pH 7.5 50 mM, NaCl 150 mM, 0.1% Nonidet P-40, EDTA pH 8 1 mM, 0,25% gelatin, 0.02% NaN). After IP, total lysates were loaded on 12% acrylamide gels and blotted using nitrocellulose membranes. The primary antibody was anti-NGF M20 (1:500, Santa Cruz Biotech.), the secondary antibody was goat anti-rabbit HRP-conjugated (1:500, Santa Cruz Biotech.). Blot images were acquired using a ChemiDoc system (Bio-Rad) and the optical density was quantified using ImageJ (NIH).

Oxytocin ELISA

To measure oxytocin levels in plasma, blood samples were drawn from the right ventricle of adult mice and centrifuged at 1000×g for 15 min. Oxytocin concentration was determined using a commercial kit (Fitzgerald, MA, USA).

Behavioral tasks on human subjects

Three female individuals heterozygous for the R100W (alt. R221W) mutation (mean age 55, SD = 11 years) were tested using a paradigm published in [10], and compared to the published normative control group (n = 18; 9 males; 21–33 years) in that study /or age-matched controls. (For a more complete description of human carrier phenotypes, see [11-15]. All participants gave informed consent, in accordance with the Declaration of Helsinki and approval by the local ethics committee, and were compensated for their participation.

Before scanning, individuals' discriminative and pain thresholds for heat and cold were collected. Discriminative thermal thresholds were determined by a procedure in which the thermode temperature started from a baseline of 32°C and increased (for heat) or decreased (for cold) at 1°C/s. The participants were asked to click a mouse button positioned on the right hand as soon as they perceived a change in the temperature. Similarly, pain thresholding started from a baseline temperature of 32°C and increased or decreased until the participant pressed the button to stop the ramping as soon as the stimulus was perceived as painful. Stimulation temperatures in the functional Magnetic Resonance Imaging (fMRI) experiment were based on the resulting individual thresholds.

During the experiment, a series of 24 thermal stimulations (painful and nonpainful heat and cold) was delivered on the dorsal part of the left hand in the MRI scanner. The stimuli were pseudorandomized within 2 different sequences during 2 separate runs, counterbalanced across subjects. In each trial, the duration of the stimulation was 4 s for heat and 6 s for cold. In all trials, the thermode had reached the target temperature when it was applied to the participant's hand. The interstimulus interval (ISI) varied from 10 s to 50 s. In one of the two runs, the participants were instructed to respond as quickly as possible to painful, but not to nonpainful, stimulation by pressing a button. In the other run, the participants responded only if the stimulation was nonpainful. For each participant the ratio of "hits" (correct responses to target stimulation), misses (missed responses to target stimulation) and false alarms (wrong responses) on overall trial number were calculated. For full details of the experimental paradigm, see [10].

An additional behavioral paradigm that addressed voluntary motor behavior to painful and nonpainful stimulation was tested in a larger group of R100W mutation carriers (N=12, all heterozygotes, 12 females, mean age 36.2 ± SDT 15.4) and their age-matched controls. The paradigm tested the participants' willingness to react to innocuous or painful stimuli. Thermal painful heat and nonpainful warm stimulation were applied to the dorsal part of the left hand for 4 s. The stimulation temperatures were tailored to the individual thresholds gathered prior to the experiment. During the stimulation the subjects had to continuously rate their willingness to move the left hand away using a mouse positioned on the right hand. A scale going from "no urge" to "high urge" was displayed on a computer screen. For each participant the slope value of the continuous rating was calculated on the whole stimulation duration (0-4 s) and compared across conditions and between groups. In

addition, the final urge score was extracted and compared across condition and between groups. This task allows the measurement of the voluntary urge to react to painful and non-painful stimulation, reflecting motivational-affective pain processing.

fMRI acquisition and data analysis

Data was collected on a 1.5-T Philips Intera magnetic resonance imaging (MRI) scanner with a SENSE head coil. For functional imaging, a single-shot echo-planar imaging sequence was used to acquire the whole brain (T2*-weighted, gradient echo sequence, repetition time_3000, echo time_35 ms, flipangle_90°, field of view_200× 244x128 mm). To minimize head movement, the participants' heads were stabilized with a vacuum hood filled with polystyrene balls (Vacuform Hood, Cambridge Research Systems, Cambridge, UK). Preprocessing and statistical analysis of MRI data were performed using BrainVoyager QX 2.1 (Brain Innovation, Maastricht, NE). Two dummy volumes were acquired before each scan in order to reduce possible effects of T1 saturation. Functional data were motion corrected and low-frequency drifts were removed with a temporal highpass filter (0.006 Hz). Spatial smoothing was applied with a 6-mm full width at half-maximum filter. Functional data were manually coregistered with 3-dimensional (3D) anatomical T1 scans (1 x 0.93 x 0.93 mm resolution resampled to 1 x 1 x 1 mm), on the basis of anatomical landmarks for each individual. The 3D anatomical scans were transformed into Talairach space[16] (primary motor cortex, peak Talairach coordinates -44, -28, 45; right striatum, including caudate head/putamen contralateral to stimulation, peak Talairach coordinates 21, 7, -1; left striatum, including caudate/putamen and parahippocampal regions contralateral to the response hand, peak Talairach coordinates -22, -4, 5), and the parameters for this transformation were applied to the coregistered functional data. For each participant, general linear models were created for each of the 4 runs. One predictor (convolved with a standard model of the hemodynamic response function, two gamma HRF) modeled each of the 8 conditions ("painful heat movement", "painful cold movement", "painful heat no-movement", "painful cold no-movement"; "nonpainful heat movement", "nonpainful cold movement", "nonpainful heat no-movement", "nonpainful cold no-movement"). To control for potential confounding factors related to movement we also included motion regressors in our analysis. Each predictor modeled the 1-s interval beginning with the onset of the cue during the last second of stimulation. Whole-brain random-effects contrasts were thresholded at $t = 3.2$ ($p < 0.001$), uncorrected for multiple comparisons. Regions of interest (ROIs) in Fig. 4d-f are defined by the main effect of "movement" in controls; ROIs in Fig. 4g are defined by the main effect of pain in R100W heterozygotes. All maps have been thresholded at $p < 0.001$ uncorrected.

Supplementary Materials References

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