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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Figure 1

a. Capsaicin test 2 months, ANOVA-1 ($F_{2,12} = 12.869$, $p = 0.002$), followed by Bonferroni post-hoc test ($NGF^{m/m}$ *vs.* DMSO, $p = 0.002$; $NGF^{m/m}$ *vs.* $NGF^{R100W/m}$, $p = 0.017$; HT^{R100W} *vs.* DMSO, NS). DMSO, $n = 3$; $NGF^{m/m}$, $n = 5$; $NGF^{R100W/m}$, $n = 5$. Capsaicin test 6 months, ANOVA-1 ($F_{2,13} = 49.995$, $p < 0.001$), followed by Bonferroni post-hoc test ($NGF^{m/m}$ *vs.* DMSO, $p < 0.001$; $NGF^{m/m}$ *vs.* $NGF^{R100W/m}$, $p < 0.001$; $NGF^{R100W/m}$ *vs.* DMSO, $p = 0.017$). DMSO, $n = 3$; $NGF^{m/m}$, $n = 5$; $NGF^{R100W/m}$, $n = 6$.

b. Hotplate test 2 months, Student's two-tailed *t*-test ($t = 0.126$, $p = 0.901$). $NGF^{m/m}$, $n = 11$; $NGF^{R100W/m}$, $n = 15$. Hotplate test 6 months, Student's two-tailed *t*-test ($t = 4.743$, $p < 0.001$). $NGF^{m/m}$, $n = 4$; $NGF^{R100W/m}$, $n = 9$.

c. Acetone test 2 months, Student's two-tailed *t*-test ($t = 2.445$, $p = 0.035$). $NGF^{m/m}$, $n = 6$; $NGF^{R100W/m}$, $n = 6$. Acetone test 6 months, Student's *t*-test two-tailed ($t = 2.457$, $p = 0.026$). $NGF^{m/m}$, $n = 8$; $NGF^{R100W/m}$, $n = 10$.

d. Tape removal test 2 months, Student's two-tailed *t*-test ($t = 1.261$, $p = 0.226$). $NGF^{m/m}$, $n = 6$; $NGF^{R100W/m}$, $n = 11$. Tape removal test 6 months, Student's two-tailed *t*-test ($t = 2.305$, $p = 0.042$). $NGF^{m/m}$, $n = 5$; $NGF^{R100W/m}$, $n = 8$.

e. Cotton swab test 2 months, Student's two-tailed *t*-test ($t = 0.155$, $p = 0.879$). $NGF^{m/m}$, $n = 5$; $NGF^{R100W/m}$, $n = 11$. Acetone test 6 months, Student's two-tailed *t*-test ($t = 0.050$, $p = 0.961$). $NGF^{m/m}$, $n = 8$; $NGF^{R100W/m}$, $n = 7$.

f. Capsaicin test on mice treated with NGF from gestation until P60, Student's two-tailed t-test ($t = 0.323$, $p = 0.754$). saline, $n = 5$; $NGF^{m/m}$, $n = 7$; $NGF^{R100W/m}$, Student's two-tailed t-test ($t = 2.764$, $p = 0.033$). saline, $n = 5$, NGF, $n = 4$.

h. Nerve conduction velocity, Aβ fiber peak, Student's two-tailed t test ($t = 0.435$, $p = 0.669$); Aδ fiber peak, Student's two-tailed t test ($t = 0.737$, $p = 0.470$); C fiber peak, Student's two-tailed t test ($t = 1.629$, $p = 0.120$); non-significant; $NGF^{m/m}$, n = 10; $NGF^{R100W/m}$, n = 11 nerves.

i. PGP9.5 immunofluorescence in glabrous skin, 2 months, Student's two-tailed t test ($t = 0.792$, $p = 0.473$); n = 3 for both groups. 6 months, Student's two-tailed t test ($t = 5.800$, $p = 0.002$); $NGF^{m/m}$, n = 4; $NGF^{R100W/m}$, n = 3.

j. NGF immunofluorescence in glabrous skin, Student's two-tailed t test ($t = 3.169$, $p = 0.034$); n = 3 for both groups.

Figure 2

a. Hyperalgesic response to intraplantar NGF injection, ANOVA-2 repeated measures (treatment × time interaction, $F_{8,144} = 5.785$, $p < 0.001$) followed by Bonferroni post-hoc test, $hNGF^{WT}$ *vs.* saline, *** $p < 0.001$; $hNGF^{WT}$ *vs.* $hNGF^{R100W}$, $^{###}$ $p < 0.001$, $^{##}$ $p = 0.002$; saline, *n* = 10; $hNGF^{WT}$, *n* = 11; $hNGF^{R100W}$, *n* = 9.

b. B2R expression in DRG cultures after incubation with NGF and bradykinin, ANOVA-2 (NGF × bradykinin interaction, $F_{2,45} = 3.371$, $p = 0.044$) followed by Bonferroni post-hoc test, *** $p < 0.001$; $hNGF^{WT}$-vehicle, *n* = 7; $hNGF^{R100W}$-vehicle, *n* = 8; control-vehicle, *n* = 8; $hNGF^{WT}$-bradykinin, *n* = 8; $hNGF^{R100W}$- bradykinin, *n* = 7; control-bradykinin, *n* = 8.

c. TRPV1 phosphorylation in DRG cultures after incubation with hNGF and bradykinin, ANOVA-2 (NGF × bradykinin interaction, $F_{2,47} = 9.346$, $p < 0.001$) followed by Bonferroni post-hoc test, *** $p < 0.001$, ** $p = 0.008$, * $p = 0.02$; *n* = 8 for each group.

d. Substance P release in DRG cultures in response to hNGF treatment, ANOVA-1 ($F_{2,16} = 10.501$, $p < 0.002$) followed by Student-Newman-Keuls post-hoc test, *** $p < 0.001$, * $p = 0.03$; $hNGF^{WT}$, *n* = 5; $hNGF^{R100W}$, *n* = 6; control, *n* = 6.

e. *Left*, B2R immunofluorescence in DRGs, Student's two-tailed *t* test ($t = 6.219$, $p = 0.003$); $NGF^{m/m}$, *n* = 3; $NGF^{R100W/m}$, *n* = 3. *Right*, TRPV1 immunofluorescence in DRGs, Student's two-tailed *t* test ($t = 12.455$, $p < 0.001$); $NGF^{m/m}$, *n* = 4; $NGF^{R100W/m}$, *n* = 4.

f. Morris Water Maze, latency to reach the platform, ANOVA-2 with repeated measures (main effect of "training day", $F_{8,112} = 15.600$, $p < 0.001$). $NGF^{m/m}$, *n* = 5; $NGF^{R100W/m}$, *n* = 8.

g. Morris Water Maze, latency to reach the platform, ANOVA-2 with repeated measures ("training day" × "genotype" interaction, $F_{8,123} = 2.836$, $p = 0.007$), followed by Bonferroni post-hoc test, * $p < 0.05$, *** $p < 0.001$. $mNGF^{+/+}$, *n* = 7; $mNGF^{+/-}$, *n* = 7.

h. Object Recognition test - sample phase, 2 months, Student's two-tailed *t* test ($t = 0.385$, $p = 0.704$). $NGF^{m/m}$, n = 7; $NGF^{R100W/m}$, n = 15; 6 months, Student's *t* two-tailed test ($t = 2.083$, $p = 0.058$). $NGF^{m/m}$, n = 9; $NGF^{R100W/m}$, n = 6; Student's two-tailed *t* test ($t = 2.014$, $p = 0.215$). $mNGF^{+/+}$, *n* = 6; $mNGF^{+/-}$, *n* = 9.

i. Object Recognition test - test phase, 2 months, ANOVA-2 (main effect of "object", $F_{1,43} = 19.916$, $p < 0.001$), $NGF^{m/m}$, n = 7; $NGF^{R100W/m}$, n = 15. 6 months, ANOVA-2 (main effect of "object", $F_{1,27} = 19.327$, $p = 0.002$), $NGF^{m/m}$, n = 9; $NGF^{R100W/m}$, n = 5; ANOVA-2 ("genotype" × "object" interaction, $F_{1,29} = 4.664$, $p = 0.040$) followed by Bonferroni post-hoc test ($mNGF^{+/+}$, new object *vs.* old object, $p = 0.018$; new object, $mNGF^{+/+}$ *vs.* $mNGF^{+/-}$, $p = 0.012$); $mNGF^{+/+}$, *n* = 6; $mNGF^{+/-}$, *n* = 9.

j. ChAT expression, medial septum, 2 months, Student's two-tailed *t*-test ($t = 0.958$, $p = 0.375$). $NGF^{m/m}$, *n* = 4; $NGF^{R100W/m}$, *n* = 4; 6 months, Student's two-tailed *t*-test ($t = 1.271$, $p = 0.278$; WT, *n* = 3; HT^{R100W}, *n* = 4); Student's two-tailed *t*-test ($t = 3.529$, $p = 0.010$; $mNGF^{+/+}$, *n* = 5; $mNGF^{+/-}$, *n* = 4).

Figure 3

a. *Left*, current thresholds for fear conditioning, 2 months, ANOVA-2 ("genotype" × "response" interaction, $F_{3,57} = 10.113$, $p < 0.001$), followed by Bonferroni post-hoc test, *** $p < 0.001$, ** $p = 0.007$, * $p = 0.021$; $NGF^{h/m}$, *n* = 11; $NGF^{R100W/m}$, *n* = 9. *Middle*, current thresholds for fear conditioning, 6 months, ANOVA-2 (main effect of "response", $F_{3,48} = 84.172$, $p < 0.001$); $NGF^{h/m}$, *n* = 10; $NGF^{R100W/m}$, *n* = 3.

b. Cued fear conditioning, 2 months, Student's two-tailed *t* test ($t = 2.348$, $p = 0.037$); $NGF^{h/m}$, *n* = 10; $NGF^{R100W/m}$, *n* = 4; 6 months, Student's two-tailed *t* test ($t = 10.190$, $p < 0.001$); $NGF^{h/m}$, *n* = 10; $NGF^{R100W/m}$, *n* = 9.

c. Contextual fear conditioning, 6 months, ANOVA-2 ("genotype" × "phase" interaction, $F_{1,22} = 436.453$, $p < 0.001$) followed by Bonferroni post-hoc test, *** $p < 0.001$; $NGF^{h/m}$, *n* = 6; $NGF^{R100W/m}$, *n* = 5.

d. c-Fos immunohistochemistry, 6 months, *upper row, left*, amygdala, Student's two-tailed *t* test ($t = 4.235$, $p = 0.003$); $NGF^{h/m}$, *n* = 4; $NGF^{R100W/m}$, *n* = 6; *middle left*, hippocampus, Student's two-tailed *t* test ($t = 2.485$, $p = 0.038$); $NGF^{h/m}$, *n* = 4; $NGF^{R100W/m}$, *n* = 6; *middle*, motor cortex, Student's two-tailed *t* test ($t = 12.226$, $p < 0.001$); $NGF^{h/m}$, *n* = 3;

$NGF^{R100W/m}$, *n* = 4; *middle right*, anterior cingulate cortex (ACC), Student's two-tailed *t* test (t = 2.349, p = 0.047; $NGF^{h/m}$, *n* = 4; $NGF^{R100W/m}$, *n* = 6); *right*, caudate nucleus, Student's two-tailed *t* test (t = 3.026, p = 0.039); $NGF^{h/m}$, *n* = 3; $NGF^{R100W/m}$, *n* = 3; *lower row*; primary somatosensory cortex (S1), Student's *t* two-tailed test (t = 0.526, p = 0.621); $NGF^{h/m}$, *n* = 3; $NGF^{R100W/m}$, *n* = 4.

e. Predator test, 6 months, *left*, Student's paired *t* test (t = 3.344, p = 0.012); $NGF^{h/m}$, *n* = 8; *middle left*, Student's paired *t* test (t = 21.847, p < 0.001); $NGF^{R100W/m}$, *n* = 7; *middle right*, Student's paired *t* test (t =4.117, p = 0.004); $NGF^{h/m}$, *n* = 8; *right*, Student's paired *t* test (t = 25.711, p < 0.001); $NGF^{R100W/m}$, *n* = 7.

f. c-Fos immunohistochemistry, hypothalamic VMH, Student's two-tailed *t* test (t = 0.243, p = 0.815); $NGF^{h/m}$, *n* = 4; $NGF^{R100W/m}$, *n* = 5.

g. Oxytocin immunohistochemistry, 6 months, hypothalamic PVA, ANOVA-2 (main effect of "genotype", $F_{1,18}$ = 19.464, p < 0.001), $NGF^{h/m}$ -baseline, n = 4; $NGF^{R100W/m}$ -baseline, n = 7; $NGF^{h/m}$ -fear conditioning, n = 3; $NGF^{R100W/m}$ -fear conditioning, n = 5.

Figure 4

a. Thresholds. Mann-Whitney U test between R100W carriers (N=3) and healthy participants (N=18). No significant difference. (U=25.5, p=0.8 for hot, U=16, p=0.3 for cold, U=19.5, p=0.4 for warm, U=17.5, p=0.3 for cool).

b. Urge to move ratings. Mann-Whitney U test on slopes from fitted curves between R100W carriers (N=12) and age-matched controls (N=12) (U=31, p =0.01 for hot, U=12 for warm). Mann-Whitney U test on the final VAS rating between R100W carriers (N=12) and age-matched controls (N=12) (U=23, p =0.005 for hot; U=55, p = 0.3 for warm).

c. Painful situation estimation. Mann-Whitney U test on R100W carriers (N=3) and age-matched controls (N=18), hits (U=1, p = 0.006), misses (U=1, p = 0.006), and false alarms (U=3, p = 0.13).

d. ß values primary motor cortex (M1). Controls. ANOVA-3 repeated measures (pain (pain, innocuous), temperature (heat, cold), and task (movement, no-movement), ($F_{1,17}$ = 7.78, *p* = 0.01) temperature x task interaction. ANOVA-2 repeated measures (temperature x task) separately for movement (*F* = 8.83, *p* = 0.009 temperature x pain interaction) and no-movement conditions (main effect of "pain" *F* = 39.5, *p* = 0.0000008, followed by post-hoc test; p = 0.001 painful heat > painful cold). Carriers. No significant effects.

e. ß values right striatum. Controls. ANOVA-3 repeated measures (pain (pain, innocuous), temperature (heat, cold), and task (movement, no-movement), ($F_{1,12}$ = 12.93, *p* = 0.002) task x pain interaction. ANOVA-2 repeated measures (temperature x task) separately for movement and no-movement conditions (main effect of "temperature" *F* = 9.23, *p* = 0.007, followed by post-hoc test; p = 0.01 painful heat > painful cold, p = 0.03 innocuous heat > innocuous cold). Carriers. No significant effects.

f. ß values left striatum. Controls. ANOVA-3 repeated measures (pain (pain, innocuous), temperature (heat, cold), and task (movement, no-movement), ($F_{1,17}$ = 7.112, *p* = 0.016) task x pain interaction. ANOVA-2 repeated measures (temperature x task) separately for movement and no-movement conditions (main effect of "temperature" *F* = 13.3, *p* = 0.002, followed by post-hoc test; p = 0.03 painful heat > painful cold, p = 0.001 innocuous heat > innocuous cold). Carriers. No significant effects.

g. Event related time-course. Contrast pain vs no-pain. (main effect of "pain", p < 0.001).

Supplementary figure 2

Post-natal mortality of $NGF^{R100W/R100W}$ mice, log-rank Kaplan-Meyer survival analysis (statistic value = 767.726, DF = 3, p < 0.001), followed by Bonferroni post-hoc test, *** p < 0.001.

Supplementary figure 3

a. Hot plate latency, 6 months, ANOVA-2 ("genotype" × "temperature" interaction, $F_{4,79}$ = 3.283, p = 0.017), followed by Bonferroni post-hoc test, *** p < 0.001, ** p = 0.003; $NGF^{m/m}$, *n* = 8; $NGF^{R100W/m}$, *n* = 8.

b. Threshold to respond, 6 months, Student's two-tailed *t* test (t = 2.789, p = 0.015); $NGF^{m/m}$, *n* = 8; $NGF^{R100W/m}$, *n* = 7.

Supplementary figure 4

a. Tape removal test, 2 months, Student's two-tailed *t* test ($t = 0.296$, $p = 0.771$); $NGF^{m/m}$, *n* = 6; $NGF^{R100W/m}$, *n* = 11; 6 months, Student's two-tailed *t* test ($t = 1.268$, $p = 0.225$); $NGF^{m/m}$, *n* = 8; $NGF^{R100W/m}$, *n* = 8.

b. PGP9.5 immunofluorescence, hairy skin, 2 months, Student's two-tailed *t* test ($t = 0.340$, $p = 0.751$); $NGF^{m/m}$, *n* = 3; $NGF^{R100W/m}$, *n* = 3; 6 months, Student's two-tailed *t* test ($t = 4.779$, $p = 0.004$); $NGF^{m/m}$, *n* = 4; $NGF^{R100W/m}$, *n* = 3.

Supplementary figure 5

DRG survival assay, ANOVA-1 ($F_{2,17} = 8.621$, $p = 0.003$), followed by Bonferroni post-hoc test, $hNGF^{WT}$ *vs.* control, $p = 0.006$; $hNGF^{R100W}$ *vs.* control, $p = 0.01$; *n* = 6 for each group.

Supplementary figure 6

a. NGF immunoprecipitation from HEK293 supernatant, ANOVA-1 ($F_{5,24} = 23.529$, $p < 0.001$) followed by Student-Newman-Keuls post-hoc test, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$; NGF, *n* = 5; $hNGF^{WT}$ 0.6 µg, *n* = 4; $hNGF^{R100W}$ 0.6 µg, *n* = 5; $hNGF^{WT}$ + $hNGF^{R100W}$ 0.3 µg/each, *n* = 3; $hNGF^{WT}$ 0.3 µg, *n* = 4;$hNGF^{R100W}$ 0.3 µg, *n* = 5; mock, *n* = 4.

b. NGF immunoprecipitation from cerebral cortex, Student's two-tailed *t* test ($t = 2.465$, $p = 0.031$); $NGF^{m/m}$, *n* = 6; $NGF^{R100W/m}$, *n* = 7.

Supplementary figure 7

Y maze test 2 months, Student's two-tailed *t*-test ($t = 1.793$, $p = 0.093$). $NGF^{m/m}$, *n* = 5; $NGF^{R100W/m}$, *n* = 12. Y maze test 6 months, Student's two-tailed *t*-test ($t = 1.074$, $p = 0.332$). $NGF^{m/m}$, *n* = 3; $NGF^{R100W/m}$, *n* = 4.

Supplementary figure 8

a. Elevated plus maze, 2 months, Student's two-tailed *t* test ($t = 2.182$, $p = 0.788$); $NGF^{m/m}$, *n* = 4; $NGF^{R100W/m}$, *n* = 11; 6 months, Student's two-tailed *t*-test ($t = 5.431$, $p = 0.003$; $NGF^{m/m}$, *n* = 3; $NGF^{R100W/m}$, *n* = 4).

b. Marble burying test, 2 months, Student's two-tailed *t* test ($t = 1.374$, $p = 0.212$); $NGF^{m/m}$, *n* = 4; $NGF^{R100W/m}$, *n* = 5; 6 months, Student's two-tailed *t* test ($t = 2.357$, $p = 0.043$); $NGF^{m/m}$, *n* = 4; $NGF^{R100W/m}$, *n* = 7.

c. Nest building test, 2 months, Student's two-tailed *t* test ($t = 1.038$, $p = 0.334$); $NGF^{m/m}$, *n* = 4; $NGF^{R100W/m}$, *n* = 5; 6 months, Student's two-tailed *t* test ($t = 2.482$, $p = 0.035$); $NGF^{m/m}$, *n* = 4; $NGF^{R100W/m}$, *n* = 7.

Supplementary figure 9

Three-chamber test, sociability session, ANOVA-2 (main effect of "condition", $F_{1,21} = 15.982$, $p < 0.001$); $NGF^{h/m}$, *n* = 4; $NGF^{R100W/m}$, *n* = 7.

Supplementary figure 10

Plasma oxytocin ELISA, Student's two-tailed *t* test ($t = 2.670$, $p = 0.020$); $NGF^{h/m}$, *n* = 5; $NGF^{R100W/m}$, *n* = 9.