Supplementary Material: Deletion of the brain-derived neurotrophic factor (*BDNF*) gene is associated with pain insensitivity.

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Supplementary Methods

Neurological Examinations

A detailed, standardized clinical neurological examination was performed by board-certified neurologists on all subjects. This examination evaluated mental status (tailored to cognitive ability), cranial nerves, motor (tone and strength), sensory, reflexes, and gait and coordination. Neurocognitive testing was performed as previously described[1]. Adaptive behavior was assessed with the Vineland Adaptive Behavior Scales, Second Edition[2]. Cognitive functioning was assessed using standardized tests of development or intelligence, including Wechsler tests[3,4], Mullen Scales of Early Learning[5], Stanford Binet Intelligence Scales, Fifth Edition[6], or the Differential Ability Scales, Second Edition[7], depending on the subject's age. Minor adjustments to these tests were sometimes employed to accommodate visual impairment (e.g., enlarging visual stimuli)[8].

Nerve Conduction Studies

Motor and sensory nerve conduction studies were performed using standard methodology on a Nicolet Viking Select of EDX machine (Cardinal Health, Dublin, OH) and were compared with published[9] and department-based normative values[10] to assess peripheral nerve function and to screen for peripheral neuropathy. The following measurements were performed: right sural sensory nerve action potential, right peroneal compound

motor action potential with F-wave latency, right median sensory nerve action potential, right median compound motor action potential with F-wave latency.

Quantitative sensory testing of human subjects with WAGR syndrome.

Testing was performed with the Medoc Thermal Sensory Analyzer (Medoc Ltd. Advanced Medical Systems, Durham, NC) using a modified-protocol derived from a prior study conducted at the NIH in adults[11]. A 1.6 x 1.6 cm contact thermode was applied to 6 different defined sites on the volar forearm within an area of approximately 4 x 10 cm. The contact thermode was held in place by the subjects so they could terminate the stimulus of their own volition. The right arm was tested first, then the left arm. The baseline adapting temperature of the thermode was 32°C with a potential range of temperatures from 0-50°C, which had been demonstrated to be a safe range in a prior study of children age 6-14y[12]. The rate of temperature change was 6°C/sec and the target temperature was maintained for 5 sec before returning to baseline. Subjects, who were masked with regard to the temperature of the stimulus, were asked to rate the thermal pain intensity of each target temperature using a 6-point Wong-Baker FACES Pain Rating Scale which has been validated in children as young as age 3 years[13]. Each temperature was tested twice on each arm, and the average of ratings of these 4 replicates for each temperature was used for analyses. For the assessment of cold pain sensitivity there were 8 target temperatures between 2-29°C, with each target temperature tested 4 times using the following algorithm of target temperatures: 29°C, 14°C, 12°C, 10°C, 8°C, 6°C, 4°C, 2°C, 14°C, 10°C, 4°C, 8°C, 2°C, 12°C, 6°C, 29°C, 12°C, 29°C, 8°C, 14°C, 2°C, 10°C, 6°C, 4°C, 2°C, 4°C, 6°C, 8°C, 10°C, 12°C, 14°C, and 29°C. For the assessment of heat pain sensitivity there were 8 target temperatures between 35-49°C ,

with each target temperature tested 4 times, using the following algorithm of target temperatures based on established protocols[14]: 35°C, 43°C, 44°C, 45°C, 46°C, 47°C, 48°C, 49°C, 43°C, 45°C, 48°C, 46°C, 49°C, 44°C, 47°C, 35°C, 44°C, 35°C, 46°C, 43°C, 49°C, 45°C, 47°C, 48°C, 49°C, 48°C, 47°C, 46°C, 45°C, 44°C, 43°C, and 35°C. The experimenter administering the test to WAGR subjects was not informed of the patient's *BDNF* deletion status, and efforts were made to blind the experimenter to *BDNF* deletion status. However, experimenters could have been cued to the subject's genotype by phenotypic presentations[15], as well as familiarity with medical records associated with each patient, which contained information about deletion boundaries of the patients, but not *BDNF* deletion status directly. Experimenters were also involved in the clinical care of these subjects.

Genotyping of *Bdnf*+/+ and +/- rats

Rats were genotyped by two separate methods in an unbiased manner. Frozen liver samples were collected and sent to TransnetYX for genotyping to detect the 6 bp deletion. Genotyping was performed using real-time PCR with a TaqMan reporter probe, and the following primers: Fwd GATGCCGCAAACATGTCTATGAG; Rev CCACTCGCTAATACTGTCACACA; Reporter CCCCGCCCGCCGTG. All animals corresponded to the expected genotype as determined by the vendor (SAGE labs) before shipment of the animals. RNA-Seq analyses were performed on the DRG in 5/10 animals of each genotype, and on the dorsal spinal cord for 10 animals. Using a grep-based strategy, reads were extracted surrounding the deletion, identifying all but one animal as the expected genotype based on the absence or presence of reads containing the deletion. This classification is imperfect due to the possibility that an mRNA containing the

deletion may not be detected if the coverage is poor. For the one animal for which RNA-Seq did not detect reads containing the deletion, three samples of the animal's liver were sent to TransnetYX, all three of which confirmed the correct genotype. This confirms that misclassification by this method was due to lack of coverage at the deletion locus. Overall transcript levels of *Bdnf* did not differ between *Bdnf*+/+ and *Bdnf*+/- animals, suggesting that the mutant transcript is stable (Supplementary Figure 4).

References

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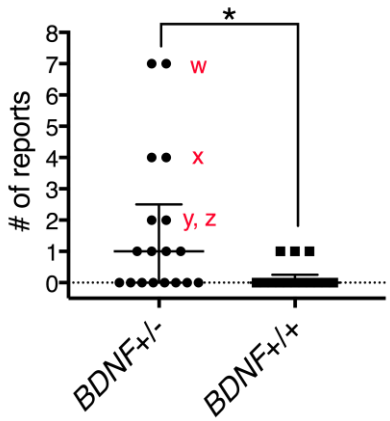
Nerve Conduction	*BDNF*+/+ (n=6)	*BDNF*+/- (n=6)	p-value
Peroneal Motor Amp (mV)	3.0 [3.0-3.4]	3.4 [2.7-3.9]	0.79
Peroneal Motor CV (m/s)	49.5 [45.0-53.0]	51.0 [47.0-58.0]	0.61
Median Motor Amp (mV)	10.9 [6.7-16.3]	11.5 [9.4-16.4]	0.66
Median Motor CV (m/s)	59.0 [54.0-60.0]	58.0 [52.0-62.0]	0.84
Median Sensory Amp (µV)	42.0 [30.0-85.0]	51.5 [16.0-70.0]	0.66
Median Sensory CV (m/s)	56.0 [50.0-68.0]	59.0 [56.0-70.0]	0.43
Sural Sensory Amp (mV)	18.0 [9.0-22.0]	19.5 [10.0-34.0]	0.57
Sural Sensory CV (m/s)	49.5 [42.0-59.0]	50.0 [48.0-68.0]	0.59

Supplementary Table 1. Nerve conduction measurements in the sub-cohort of WAGR patients who completed quantitative sensory testing. Measurements were performed on the right side for all subjects. Abbreviations: conduction velocity (CV), millivolts (mV), meters/second (m/s). Median [range] shown with nominal *p*-values from Mann-Whitney U tests. Demographic information is in Figure 1.

Nerve Conduction	*BDNF*+/+ (N=12)	*BDNF*+/- (N=20)	*p*-value
Age	13 [6-28]	11.5 [6-37]	0.69
Sex (% female)	66.7	50	0.47
Peroneal Motor Amp (mV)	3.0 [1.8-4.3]	3.4 [1.3-8.8]	0.54
Peroneal Motor CV (m/s)	48 [45.0-53.0]	49 [29-58]	0.60
Median Motor Amp (mV)	10.9 [5.2-16.3]	9.15 [5.1-16.4]	0.89
Median Motor CV (m/s)	60 [45-69]	56 [50-63]	0.12
Median Sensory Amp (μV)	42 [20-85]	47 [12-73]	0.80
Median Sensory CV (m/s)	58 [47-68]	60 [52-70]	0.56
Sural Sensory Amp (mV)	14.5 [6-27]	18 [5-34]	0.31
Sural Sensory CV (m/s)	49 [42-59]	50 [40-64]	0.51

Supplementary Table 2: Nerve conduction studies in the *BDNF*+/+ and *BDNF*+/- subjects. Measurements were performed on the right side for all subjects. Abbreviations: conduction velocity (CV), millivolts (mV), meters/second (m/s). Median [range] shown with nominal *p*-values from Mann-Whitney U tests. Demographic information for this cohort can be found in a previously published report.[15]



Supplementary Figure 1 – Summary of parental reports of pain insensitivity in *BDNF*+/+ and *BDNF*+/- WAGR individuals. Parents of patients with WAGR syndrome were surveyed using the Non-Communicating Children's Pain Checklist-Revised (NCCPC-R)[16] to retrospectively assess responses to typically painful injuries and illnesses. Comments were an optional component of the survey, which some parents completed minimally while others elaborated extensively. Parents of *BDNF*+/- patients had significantly more descriptions of their child's pain sensitivity. The large number of zero values is reflective of the fact that these responses were optional, and not all parents filled them out in with the same degree of detail. Excerpts are included below. Two-tailed Mann-Whitney U test, *, $p < 0.05$; N=18 *BDNF*+/-, N=14 *BDNF*+/+. Bars represent median with interquartile range.

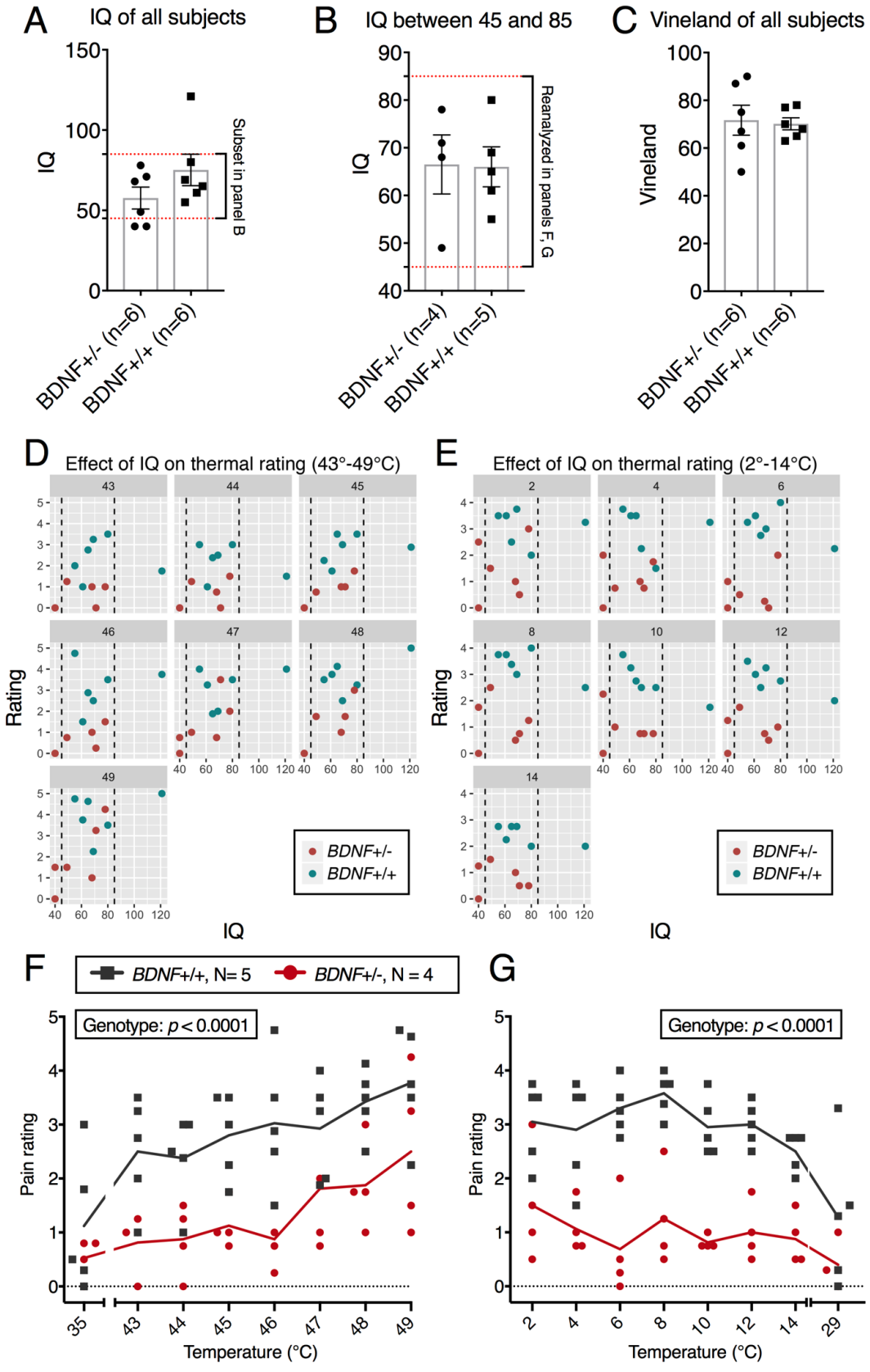
Excerpts from parental reports:

w. "She has extremely high tolerance for pain. It has to be extraordinarily painful and fortunately, pain of this degree does not happen frequently. Examples of this type pain: Fractured pelvis, sepsis UTI infection"

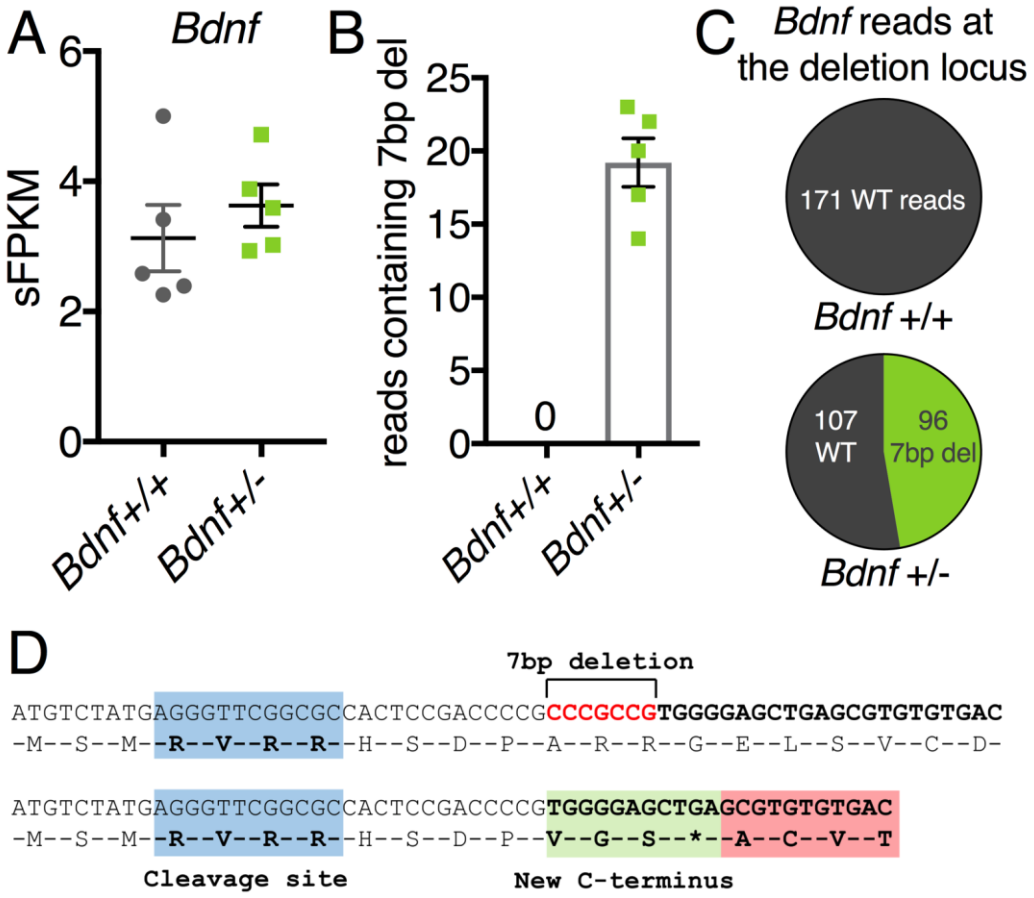
x. "Has mashed his finger in a heavy glass commercial door, turning it immediately blue, and has shown no response, EXCEPT to put his other finger in the door! He has dislocated his knee. The day after a nephrectomy, he got up out of bed and walked as if nothing had happened."

y. "Got his toe caught in our gate and it ripped a big 1/2" gouge in it. It was bleeding pretty heavily and the skin was just hanging off. He also broke a bone in his hand while riding a bike, we didn't notice the bruise until the next day"

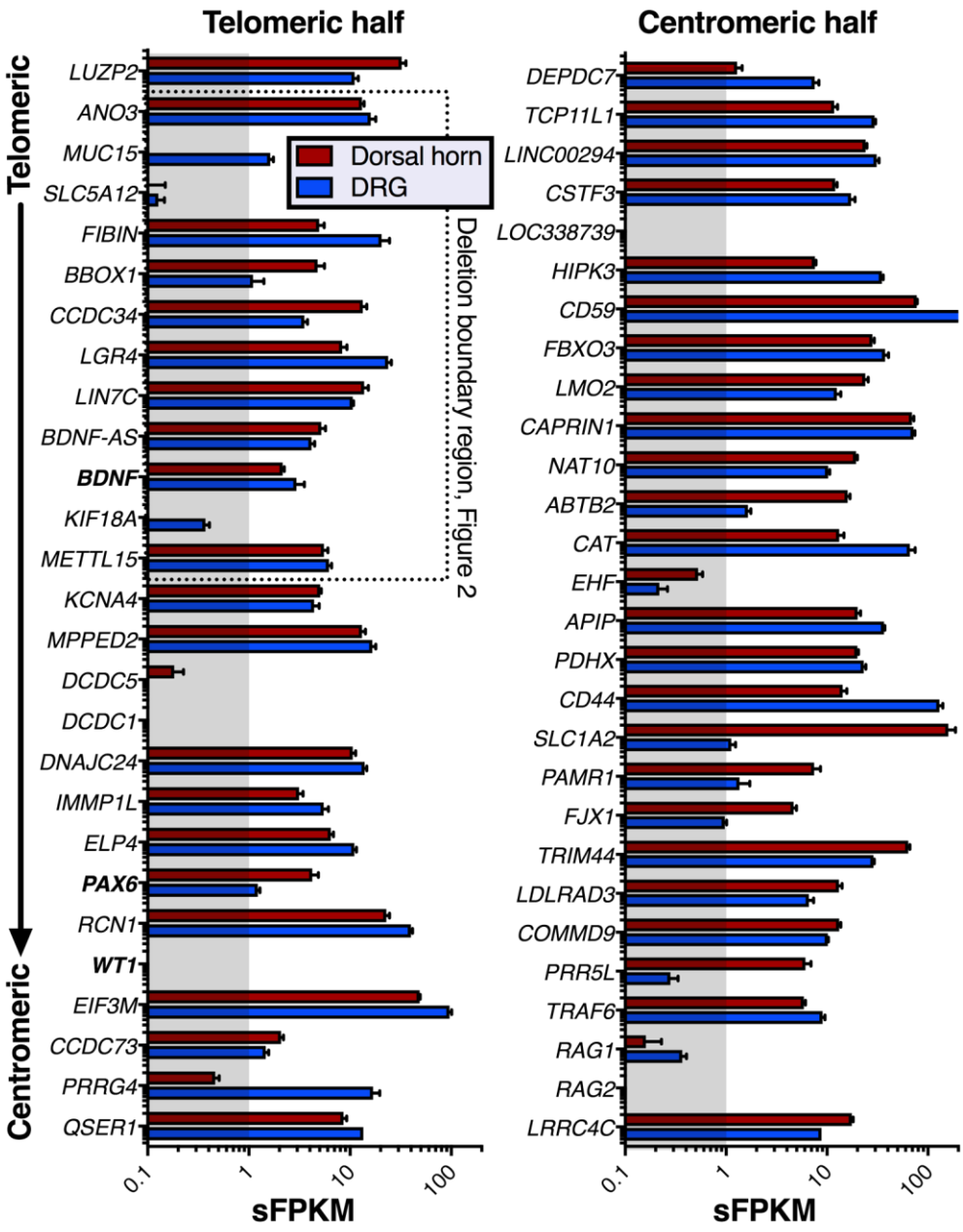
z. "But I believe there are many times when I know nothing. She had a punctured ear drum once that I only discovered from the discharge coming out. Took her to the doctor and asked her if her ear hurt and she said, 'not really.'"


A
IQ of all subjects
IQ
150
100
50
0
Subset in panel B
BDNF+/- (n=6)
BDNF+/+ (n=6)
B
IQ between 45 and 85
IQ
90
80
70
60
50
40
Reanalyzed in panels F, G
BDNF+/- (n=4)
BDNF+/+ (n=5)
C
Vineland of all subjects
Vineland
100
80
60
40
20
0
BDNF+/- (n=6)
BDNF+/+ (n=6)
D
Effect of IQ on thermal rating (43°-49°C)
43
44
45
46
47
48
49
Rating
IQ
BDNF+/-
BDNF+/+
E
Effect of IQ on thermal rating (2°-14°C)
2
4
6
8
10
12
14
Rating
IQ
BDNF+/-
BDNF+/+
F
BDNF+/+, N= 5
BDNF+/-, N = 4
Genotype: p < 0.0001
Pain rating
Temperature (°C)
35
43
44
45
46
47
48
49
G
Genotype: p < 0.0001
Pain rating
Temperature (°C)
2
4
6
8
10
12
14
29

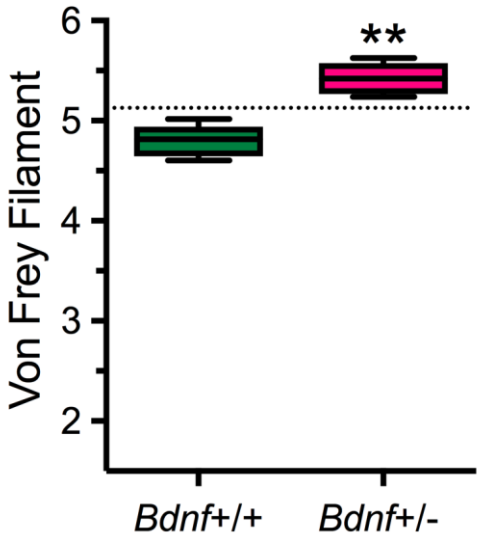
Supplementary Figure 2 – Relationship between IQ and thermal rating of hot and cold stimuli in WAGR subjects. WAGR subjects who participated in QST (N=6 in each genotype) were examined for differences in IQ. **A.** IQ of the subjects in the study is plotted, showing that between IQ of approximately 45-85, the variance between groups is reduced. **B.** Three subjects outside the 45-85 IQ range were excluded for a sensitivity analysis to equalize mean IQ between groups. **C.** Vineland adaptive behavior scale values do not show any difference between groups. **D, E.** Thermal ratings from Figure 1D, E are plotted against IQ within each temperature, showing the relationship between IQ and rating. Dotted lines indicate the IQ range selected for sensitivity analysis (45-85), indicating the degree to which the samples outside this range may skew the distributions. **F, G.** The same graph from Figure 1D, E is shown with the three excluded samples removed, and the same statistical test was applied. In this reanalysis, the genotype effect (BDNF+/- vs BDNF+/+) is still highly significant ($p < 0.0001$) in both cases. Error bars in A, B, C show the standard error of the mean.



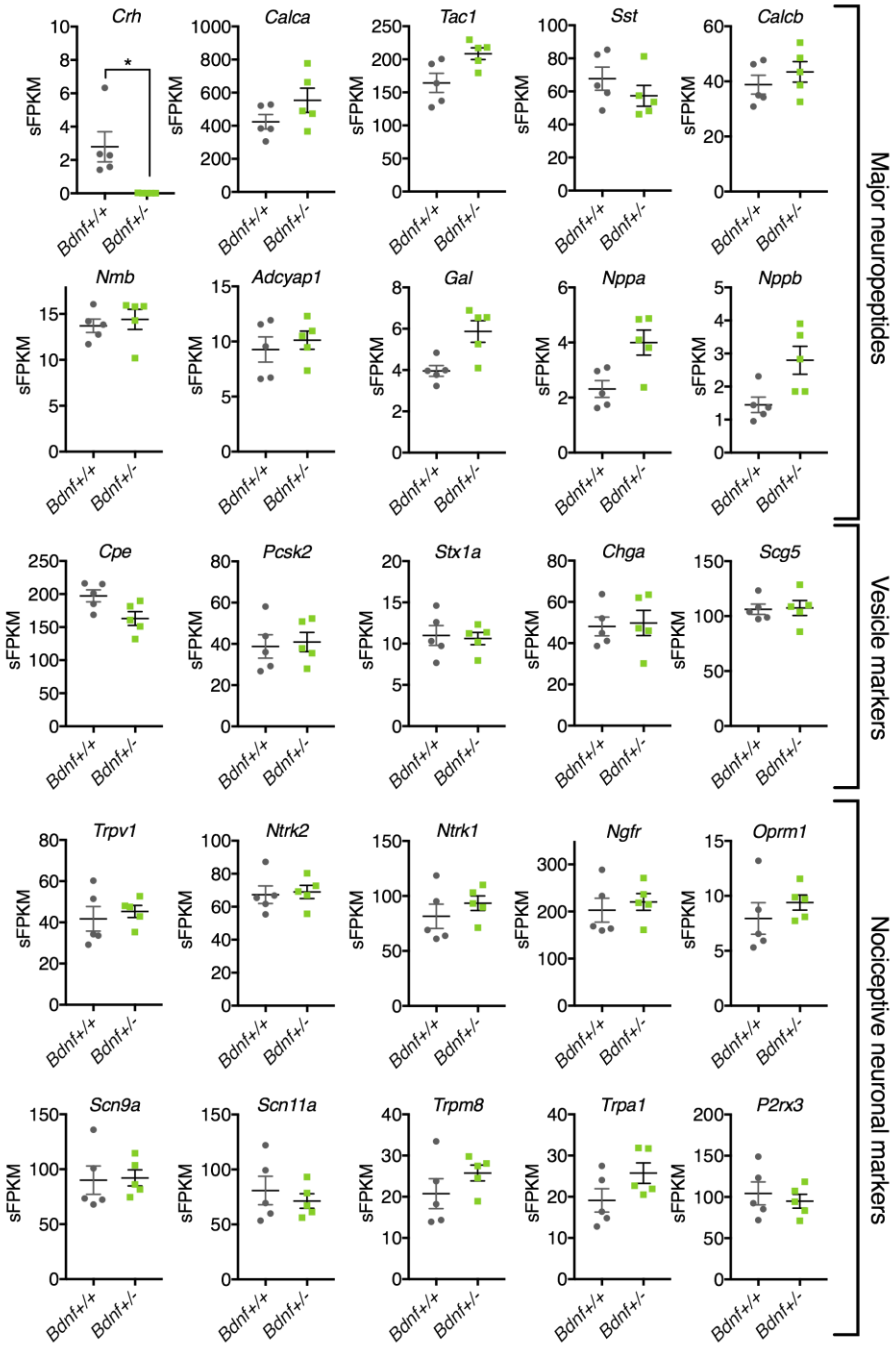
Supplementary Figure 3. Genotyping and expression analysis based on DRG sequencing. (A) Rats of each genotype in the present study express comparable amounts of either wildtype or mutant *Bdnf* transcript (N=5, $p = 0.3095$ two-tailed Mann-Whitney test) with (B) reads containing the 7 bp deletion locus present in *Bdnf*+/-, but not *Bdnf*+/+ animals ($p = 0.0079$ two-tailed Mann-Whitney test). (C) Approximately half of the *Bdnf* transcript expressed by the *Bdnf*+/- animals contains the expected 7 bp deletion. (D) The deletion leads to frameshift mutation followed by an early stop codon (asterisk). This occurs four codons 3' of the sequence encoding the propeptide furin-like cleavage site (blue), resulting in loss of translation of the BDNF peptide.



Supplementary Figure 4. RNA-Seq examinations of human dorsal horn and DRG expression of genes deleted in WAGR subjects. Patients in the present study had variable deletion boundaries. Well-annotated genes within the deletion boundaries of the subjects in the current study were examined in control human dorsal horn and DRG tissues, to search for genes potentially involved in nociception. Many of the genes in the deletion locus are expressed in control human DRG and spinal cord. For this analysis, one sFPKM is considered the cutoff for meaningful expression (gray bar). The majority of the genes in this locus are expressed in DRG and/or spinal cord tissue, indicating their potential for involvement in nociceptive circuits. The critical genes expressed at the boundary between the two groups (Figure 2) are outlined (dotted line).

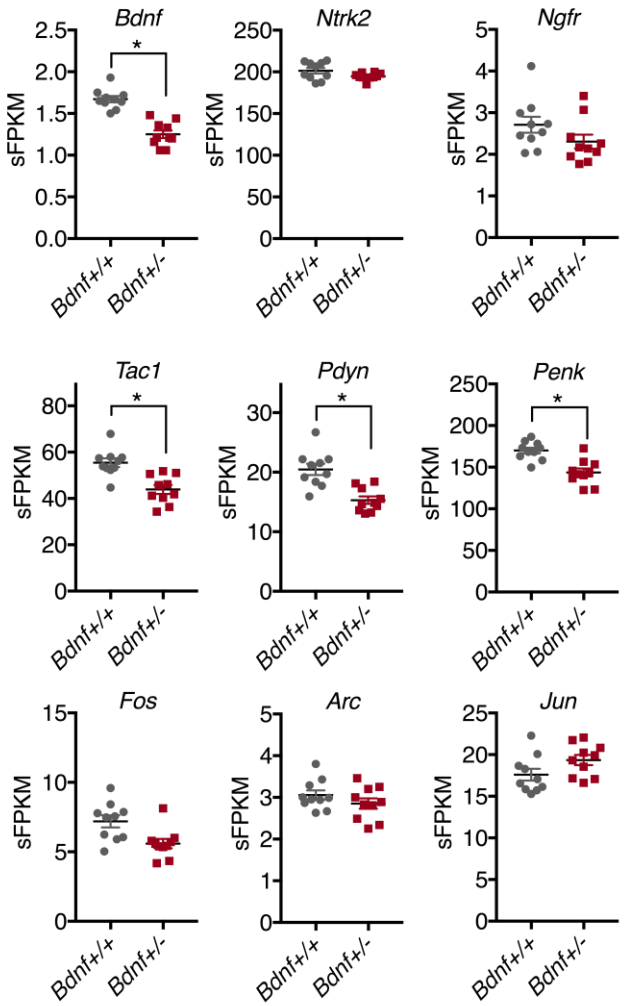


Supplementary Figure 5. Von Frey testing in *Bdnf*+/+ and *Bdnf*+/- rats. Animals were tested for mechanical sensitivity by von Frey filament withdrawal using the up-down method. Error bars represent the range of data points, and a dotted line is inserted to show total separation between the filament sufficient to provoke a response between animals. **, $p<0.01$ Mann-Whitney U test.

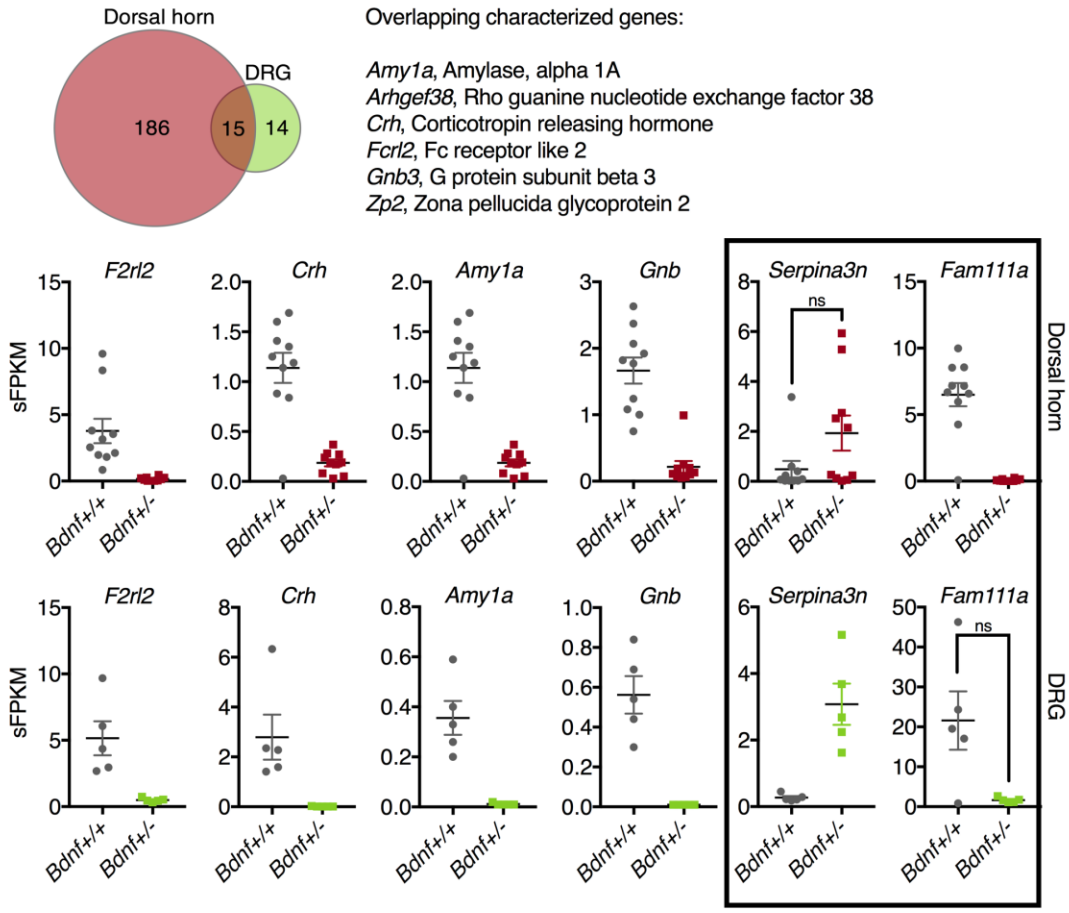


Supplementary Figure 6. Gene expression panels in *Bdnf*+/+ and *Bdnf*+/- DRG. In order to examine the effects of *Bdnf* haploinsufficiency on DRG gene expression, RNA-Seq was performed, and panels of marker genes were individually examined (N=5). The gene encoding the peptide precursor for Corticotrophin releasing hormone (*Crh*), although not highly expressed, was among the strongest genes decreasing in *Bdnf*+/- animals. Numerous other neuropeptide precursor genes showed mild non-significant trends towards increasing or decreasing. However, markers of vesicles or of neuronal cells themselves were unchanged, suggesting that the same types of cells were present in

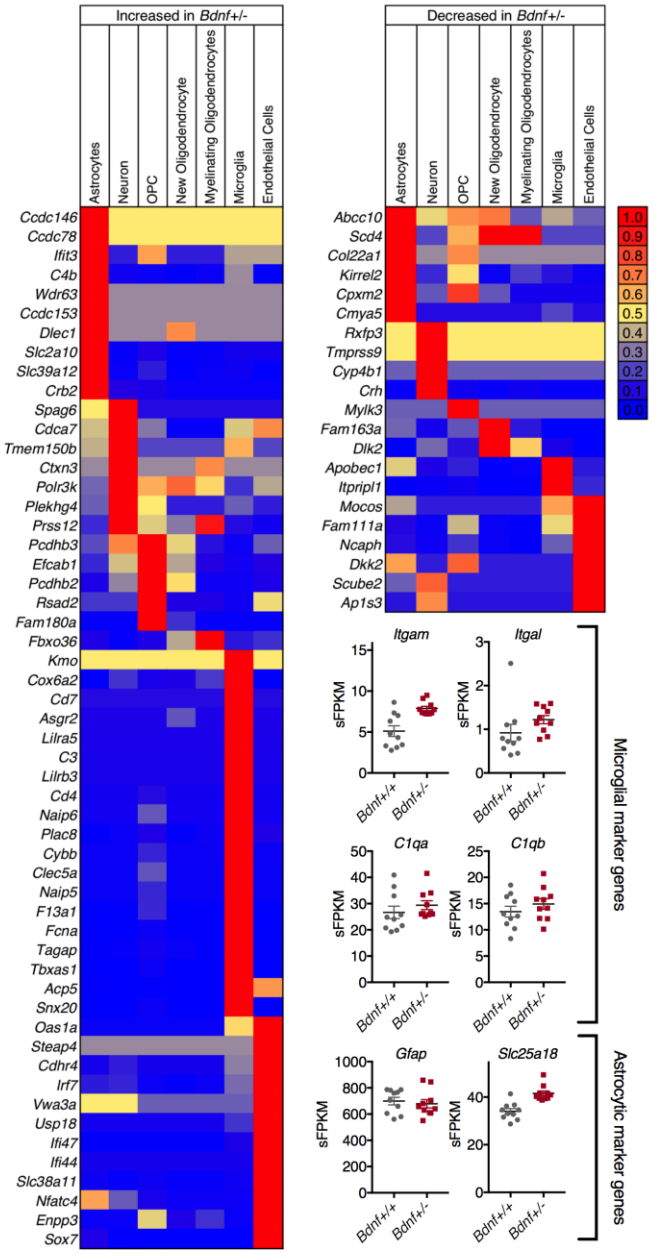
the DRG of both genotypes. We cannot rule out a difference in signaling or connectivity that might lead to altered production of neuropeptides.



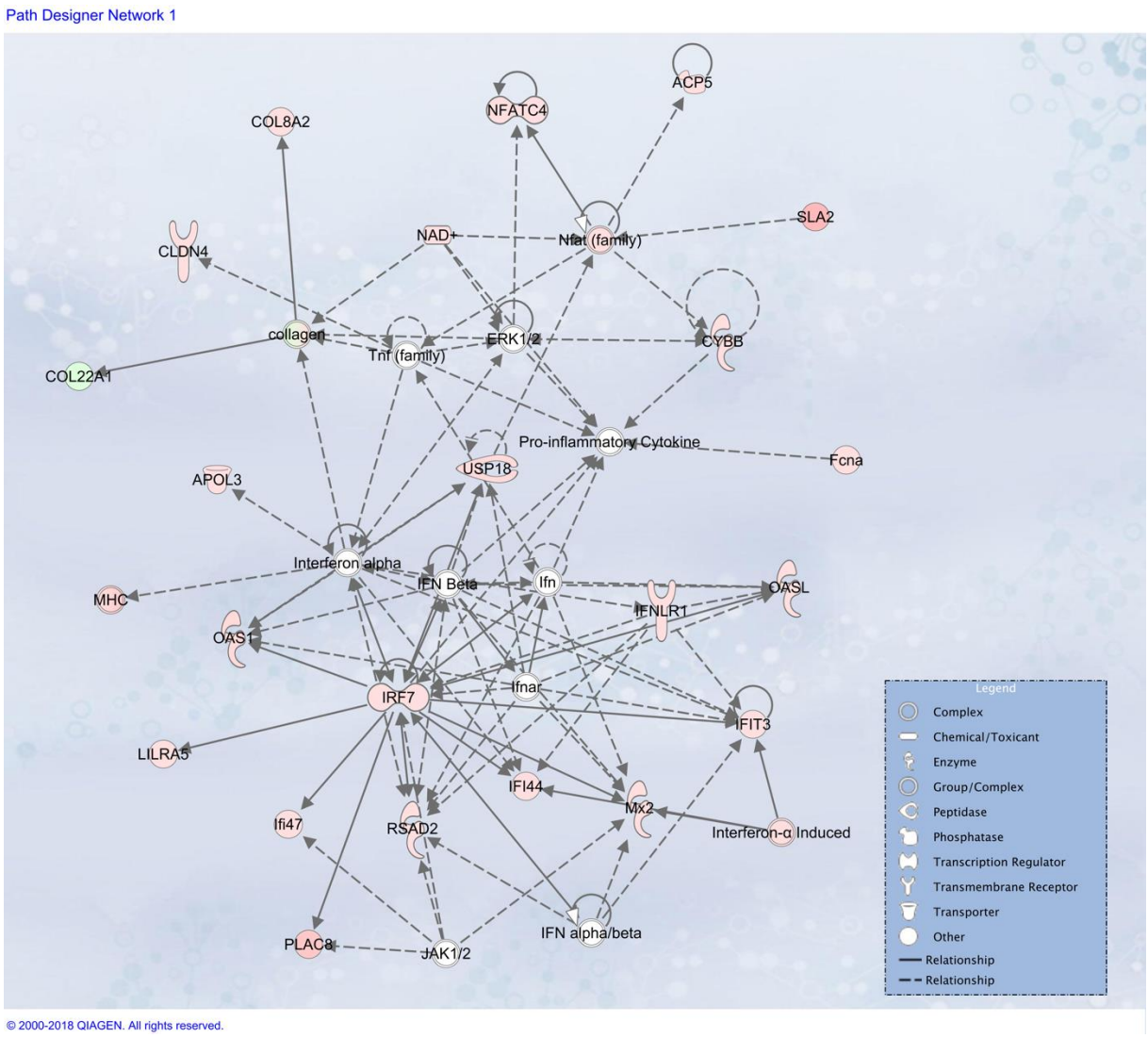
Supplementary Figure 7. Selected subset of dorsal horn genes in *Bdnf*+/- rat dorsal spinal cord. In a preliminary analysis of dorsal horn genes, we selected a subset of genes of interest, including *Bdnf* itself. Because of the small magnitude of gene changes in the dorsal root ganglia and the general trend towards mild effects on neuropeptide precursor genes, we selected a subset to examine by two-tailed Mann-Whitney U-test to look for smaller changes in genes expected to be informative. In these analyses, several neuropeptides showed significant, small magnitude changes, suggesting subtle modulation of peptidergic signaling in the dorsal horn of *Bdnf*+/- rats. Three major neuropeptides involved in pain signaling, preprotachykinin, and the opioid peptide precursors preprodynorphin and enkephalin were downregulated, as was *Bdnf*. These genes are not among the genes identified by the FDR-based significantly differential gene selection performed in MAGIC[17], which controls for multiple comparisons across all genes measured in these tissues.



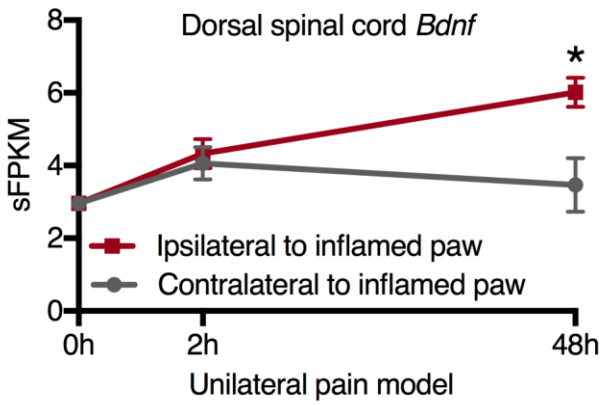
Supplementary Figure 8. Genes altered in both *Bdnf*+/- dorsal spinal cord and DRG. Differential genes between *Bdnf*+/+ and *Bdnf*+/- rats were calculated in MAGIC for both DRG and dorsal spinal cord tissues. In general, the dorsal spinal cord showed more differentially expressed genes (201) compared to the DRG (29). Of these, 15 genes were differential in both tissues. Characterized overlapping genes with gene names are listed. The 4 highest expressed genes are shown with detailed levels of expression for spinal cord (middle panel) and DRG (lower panel). The genes expression decreases in the BDNF +/- animals in both nervous system tissues. The remaining genes are uncharacterized loci, or expressed at very low levels. *Serpina3n* and *Fam111a* were significant in only one tissue, but showed a trend in the same direction in the other tissue, and were among the most highly expressed differential genes.



Supplementary Figure 9. Gene expression panels in *Bdnf*+/+ and *Bdnf*+/- DRG. Differentially expressed genes (MAGIC) in the dorsal horn of Bdnf+/- rats were compared to a database of neuronal and non-neuronal cell types. The majority of these genes that were increased in Bdnf+/- rat dorsal spinal cord were enriched in astrocytes or microglia. General markers of these cell types were examined to investigate whether these cell types were proliferating or infiltrating, but these general markers were not altered, suggesting more subtle gene regulation within these cells as opposed to change in their overall tissue contribution.



Supplementary Figure 10. Upregulation of genes in the interferon pathway, and identification of interferon-related genes as downstream hubs using pathway analysis. Pathway analysis was performed in IPA (Qiagen) using the geneset from the RNA-Seq data regulated in response to *Bdnf* heterozygous deletion. The regulation of several genes points to the interferon pathway as a central hub for downstream effects of *Bdnf* deletion.



Supplementary Figure 11. Regulation of *Bdnf* transcript expression by peripheral inflammation. N=6 rats were inflamed with carrageenan injection into the plantar surface of the hindpaw, and the dorsal quadrant of the spinal cord was dissected and processed for RNA-Seq analyses. The *Bdnf* transcript was strongly regulated by peripheral inflammation, showing induction at the 2h and 48h time points. Significance values were calculated in MAGIC using the FDR method[17,18].