Published in final edited form as:

Pain. 2019 May ; 160(5): 1070–1081. doi:10.1097/j.pain.0000000000001485.

Haploinsufficiency of the brain-derived neurotrophic factor (*BDNF*) gene is associated with reduced pain sensitivity.

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Abstract

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Author Contributions

M.R.S. designed experiments, performed behavioral testing and RNA-Seq analyses on animals, performed statistics, constructed figures, and analyzed and interpreted the data. D.M.L. analyzed the data, constructed figures, and extracted RNA. T.L. performed and interpreted nerve conduction measurements for human subjects. A.E.T. performed and interpreted cognitive testing in human subjects, and assisted in designing and interpreting the pain assessments for human subjects. K.M.D. S.R.F. M.D.L. A.E.H. directly performed or supervised research assistants in pain testing of human subject. S.J.S. and J.W.T. performed and interpreted neurological examinations on human subjects. M.J.I. analyzed the data, supervised behavioral assessments. J.C.H. designed human pain testing methods adapted for visual impairment, performed statistics, and analyzed the human subject data. M.J.I., J.A.Y., A.J.M., and J.C.H. supervised the overall project and management. M.J.I., J.A.Y., and J.C.H. conceived the project. M.R.S., M.J.I., and J.C.H. prepared the manuscript.

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The authors have no conflicts of interest at the time this manuscript was written.

Rare pain insensitive individuals offer unique insights into how pain circuits function, and have led to the development of new strategies for pain control. We investigated pain sensitivity in humans with WAGR (Wilms tumor, aniridia, genitourinary anomaly, range of intellectual disabilities) syndrome, who have variably-sized heterozygous deletion of the 11p13 region. The deletion region can be inclusive or exclusive of the brain-derived neurotrophic factor (*BDNF*) gene, a crucial trophic factor for nociceptive afferents. Nociceptive responses assessed by quantitative sensory testing (QST), demonstrated reduced pain sensitivity only in the WAGR subjects whose deletion boundaries included the *BDNF* gene. Corresponding behavioral assessments were made in heterozygous *Bdnf* knockout rats to examine the specific role of *Bdnf*. These analogous experiments revealed impairment of Aδ and C-fiber mediated heat nociception, determined by acute nociceptive thermal stimuli, and in aversive behaviors evoked when the rats were placed on a hot plate. Similar results were obtained for C-fiber mediated cold responses and cold avoidance on a cold plate device. Together, these results suggested a blunted responsiveness to aversive stimuli. Our parallel observations in humans and rats show that heterozygous deletion of the *BDNF* gene reduces pain sensitivity, and establish BDNF as a determinant of nociceptive sensitivity.

Summary:

Haploinsufficiency of *BDNF* in humans with WAGR syndrome, and in rats with a specific disruption of *Bdnf* on a single allele have reduced sensitivity to pain.

Keywords

BDNF; pain sensitivity; nociception; nerve growth factor; RNA-Seq

Introduction

The ability to sense and respond to potentially damaging stimuli is a fundamental role of the nervous system, conserved in all animals. Dedicated peripheral sensory neurons, called “nociceptors” respond to tissue damage, transducing the stimuli into neural impulses, and are the first neuron in the pain pathway. However, in the central nervous system the registration and interpretation of these sensory inputs involves complex circuitry some of which feeds back to modulate primary afferent nociceptive inputs in dorsal spinal cord[47]. Although there is variation in pain sensitivity across normal individuals, there are also rare outliers; at one extreme are people with congenital insensitivities to pain. Such people generally harbor mutations which inactivate or destroy the nociceptive apparatus in the peripheral nervous system, leading to profound loss of pain sensation[13; 50]. Such rare mutations have led to a greater understanding of pain transmission, development of pain circuits, and ultimately to new approaches to control pain[16; 25].

The present report focuses on patients with Wilms tumor-aniridia (WAGR) syndrome which is caused by a variable-length heterozygous deletion in 11p13, and is associated with a clinical heterogeneity and a large number of phenotypic presentations that include kidney tumors (nephroblastoma), aniridia, genitourinary anomalies (e.g., cryptorchidism), and intellectual disabilities. In part, clinical heterogeneity is driven by the variable genetic defect, that can be inclusive or exclusive of several genes, including the brain-derived

neurotrophic factor (*BDNF*) gene. Based on parental reports of pain insensitivity in WAGR syndrome patients with *BDNF* deletion on one chromosome, as well as a large existing literature on the relationship between BDNF and pain [38; 40; 47], we systematically investigated pain sensitivity in individuals with WAGR syndrome. We show that haploinsufficiency of the *BDNF* gene is associated with a strong reduction in pain sensitivity in these individuals that was evident using quantitative sensory testing (QST) and via parental reports. Similarly, a rat model that specifically isolates the *Bdnf* haploinsufficiency also revealed impairment of cold and hot thermo-nociception. In our transcriptomic examinations of the first two elements of the nociceptive circuit, DRG and dorsal spinal cord, we see more genes differentially regulated at the level of the second order spinal neurons, suggestive of pain modulation, rather than complete abolition of the primary afferent nociceptive apparatus. Our observations in humans and rats establish corresponding phenotypic evidence in both species that *BDNF* haploinsufficiency is associated with altered nociceptive sensitivity, and have potential implications for future pharmacologic modulation of pain sensitivity.

Materials and Methods

Subjects.

Subjects with WAGR syndrome were recruited through the International WAGR Syndrome Association. All procedures were approved by the NICHD Institutional Review Board. Parents/legal guardians provided consent for minors and adults with intellectual disability. Testing was performed at the NIH Clinical Research Center in Bethesda, Maryland, USA. A detailed, standardized clinical neurological examination was performed by board-certified neurologists on all subjects.

Demographics, neurological examination, and genotyping of WAGR subjects.

11p13 deletion boundaries for the WAGR subjects were determined by microarray comparative genomic hybridization[23]. The mapping of the WAGR hemideletion allowed the patient population to be split based on deletion boundaries. Out of the 12 patients in the present study, 6 harbored heterozygous deletion of *BDNF*, while 6 retained two copies of this gene (Figure 1A). These two groups of patients were not significantly different in intelligence, age, tumor status, chemotherapy treatment (Figure 1B), or in nerve conduction velocity (Supplementary Tables 1, 2). Both groups of patients were 2/3 female. Parental reports of these patients indicated a potential pain insensitivity phenotype, and were recorded formally (Figure 1C, further annotated in Supplementary Figure 1), and investigated with experimental thermal pain stimuli using QST (Figure 1D,E). Most WAGR syndrome subjects are intellectually impaired, and deletion of *BDNF* is associated with reduced general cognitive functioning[24]. Due to this impairment, criteria for eligibility included ability to rate thermal stimuli and complete QST. Sensitivity analysis was performed to rule out IQ as a confounding variable in the interpretation of QST results (Supplementary Figure 2). In addition, clinical neurological examination demonstrated normal peripheral motor and sensory findings consistent with the normal conduction velocity measurements on multiple peripheral nerves. Nerve conduction measurements are shown for the cohort in Supplementary tables 1 and 2.

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 1.6×1.6 cm contact thermode placed on the volar forearm. This protocol was derived from a prior study conducted at the NIH in adults [33]. 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 [59]. 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 [23], 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.

Animal experiments were conducted under a protocol approved by the Clinical Center Animal Care and Use Committee. 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 7 bp deletion (SD-*Bdnf*^{tm1sage}, Horizon Discovery, Cambridge, UK). Genotyping was performed using real-time PCR with a TaqMan reporter probe, and the following primers: Fwd GATGCCGCAAACATGTCTATGAG; Rev CCACTCGCTAATACTGTCACACA; Reporter CCCC GCCCGCCGTG. All animals corresponded to the expected genotype as determined by the vendor (SAGE Labs) before shipment of the animals. Two cohorts of N=5 per group male *Bdnf*^{+/+} and *Bdnf*^{+/–} rats were assessed in the current study. Genotype was corroborated by RNA-Seq analyses 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 (SPELL THIS acronym OUT OR explain a bit for the readers), 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 the RNA-Seq 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 3).

Behavioral assessments of WT and *Bdnf*^{+/–} rats.

Radiant thermal and noxious cold stimulation.

All behavioral assessments were performed by an experimenter who was blinded to genotype at the time of testing. Sample sizes were determined based on previous experience with similar testing paradigms. Rats were enclosed in individual boxes on a 2.5 mm thick elevated glass plate, and radiant heat stimulation was applied to the plantar surface of the

hind paw using a focused infrared halogen bulb (Plantar Test, Ugo Basille, Monvalle VA, Italy) [28; 44]. Heating was terminated when the animal withdrew the hindlimb, and behavior was scored as the latency to withdraw. To determine temperature over time, the same radiant heat stimulus was delivered to awake animals while standing on a thermistor wire (type K) connected to a USB universal thermocouple connector (Omega Engineering Inc., Stamford, CT) to measure the temperature at the skin's surface. Temperature over time curves were used to convert latency measurements into temperature of withdrawal. The temperature at which withdrawal occurred was calibrated by delivering the radiant heat stimulus in an awake behaving rat in a quadrupedal stance. A thin thermistor wire (type K, Omega Engineering Inc., Stamford CT) was placed under the stimulated hind paw in order to measure temperature at the skin's surface. Temperature-over-time curves were used to convert latency measurements into temperature at which the animal withdraws.

Measurements of acute cold pain were based on a similar method published previously [7]. Powdered dry ice was compacted into the barrel of a 12 mm diameter plastic syringe and used to deliver a cold stimulus to the plantar surface [7]. Briefly, animals were enclosed in individual clear plastic boxes on an elevated glass platform that was 2.5 mm thick, and cold stimulation was delivered to the animal's plantar hindpaw from below. Care was taken to ensure a smooth surface of the dry ice, and light pressure was applied to make contact between the dry ice and the glass. Withdrawal latency and thermistor measurements were made in the same manner as for radiant thermal stimulation.

Hot and cold plate behavioral assessment.

To determine behavioral response to noxious heat, animals were placed on a hot plate set to 48°C until they licked their hind paw twice and latency to first and second hind paw licks were recorded [1]. This temperature was chosen because it is at the low end of the noxious range usually used to perform this test, where we hypothesized the greatest likelihood of differentiating the two groups. To assess responses to noxious cold, animals were placed on the same plate and behaviors were recorded for 5 minutes using an iPhone 6 (Apple, Cupertino, CA). Behaviors were scored manually, and values were checked for agreement by three observers, one of whom was blinded to experimental conditions. During the cold plate assay, the rats displayed avoidance behaviors by lifting their tail off the surface of the plate. The sum of the duration of avoidance episodes was determined from the video recordings by observers blinded to genotype.

A δ stimulation using 100 ms infrared diode laser pulse.

Animals were set on the same glass platform as for noxious cold stimulation, and an infrared diode laser (LASS-10 M; Lasmed, Mountain View, CA, USA) with an output wavelength of 980 nm was used to deliver 100 ms thermal stimuli of approximately 1.6 mm diameter to the heel and plantar surface of the rat hind paw. This stimulus has previously been shown to selectively activate A δ fiber-mediated nociceptors [3; 41; 56]. Laser stimulator was operated as described previously [41; 42] with an output between 3000 and 5500 mA to obtain a stimulus-response function. After delivering a stimulus, animals were scored for behavior according to the following rubric: 0 = no response, 1 = simple withdrawal, 2 = paw shake, 3 = orient towards paw, 4 = paw lick. Statistics were performed on average behavioral values

using the Mann-Whitney U-test to account for ordinal rank-orders. Response ratios were calculated by considering 0's vs all responses regardless of severity.

RNA extraction and sequencing.

RNA extraction was performed using a bead beating homogenizer (MP Biomedicals, Santa Ana, California) and the RNeasy Lipid Tissue Mini Kit as described previously [34; 46]. Sequencing was performed at the NIH Intramural Sequencing Center, as described previously [46]. Briefly, mRNA libraries were constructed starting from 1 µg total RNA using the Illumina TruSeq RNA Sample Prep Kits, version 2. The resulting cDNA was fragmented using a Covaris E210. Library amplification was performed for 10 cycles to minimize the risk of over-amplification. Unique barcode adapters were applied to each library. Libraries were pooled in equimolar ratio and sequenced together on a HiSeq 2500 with ver 4 flow cells and sequencing reagents. A minimum of 70 million 125-base read pairs were generated for each DRG library, and a minimum of 23 million 125-base read pairs were generated for each dorsal horn library.

Transcriptomic analyses.

Several datasets were mined from previous publications [48]. Mouse brain neural and non-neural cell population RNA expression data were mined from a previous database [62] and used to categorize significantly regulated genes [46]. Heatmap data were constructed from expression data mined from the human GTEx database and plotted as a ratio of expression per gene to identify tissue enrichment for genes of interest [8; 48]. Rat DRG and dorsal spinal cord datasets generated for this manuscript were aligned and quantified using MAGIC [34; 61] and a rat genome with annotations built based on rn6 [34]. Gene expression values are represented using significant fragments per kilobase of transcript per million aligned reads (sFPKM), a MAGIC-specific quantification method [34; 61]. Significantly differential genes were determined by controlling the false discovery rate at 5% after comparing against 80 orthogonally scrambled iterations of sample groupings to control for variance [34; 46]. In the dorsal horn dataset, which was collected after the DRG dataset, several candidate genes were examined using uncorrected Mann-Whitney U-tests based on previous observations in DRG. Gene expression and fold changes were analyzed for interactions using Ingenuity Pathway Analysis (Qiagen).

Data availability.

Rat sequencing data from the DRG and dorsal horn of *Bdnf*^{+/+} and *Bdnf*^{+/-} animals has been deposited into the Sequence Read Archive under BioProject number (To be released upon publication of this manuscript).

Statistical analyses.

Linear mixed-effects models were constructed in SAS 9.4 (SAS Institute Inc., Cary, NC) for human sensory testing data (Figure 1D, E) using rating, temperature, baseline response, and IQ as fixed effects, with individual as a random effect. In these models, temperature was considered as a factor to account for nonlinearity in temperature ratings. Sensitivity analysis was performed to examine the effect of IQ on thermal ratings from QST (Supplementary

Figure 2). A similar linear mixed-effects model was constructed for the cold plate assay (tail lifting) using temperature and genotype as fixed effects, and animal number as a random effect. For all models, least square means tests are reported for each measurement. Mann-Whitney U-tests and Student's T-tests were performed in Prism 7 (GraphPad Software, LaJolla, CA). Full results from all statistical tests are reported in the supplementary data files. For RNA-Seq datasets, statistical analyses were performed using MAGIC [34]. GO terms and related statistics were calculated in DAVID 6.8 (<https://david.ncicrf.gov/>).

Results

Parental reports.

Parents of *BDNF*^{+/-} WAGR subjects were significantly more likely to provide descriptions of their child's pain insensitivity in the optional comments section of the Non-Communicating Children's Pain Checklist-Revised [6; 23] (Figure 1C, Supplementary Figure 1). These reports contained striking details of severely reduced pain sensitivity resembling those observed in cases of complete pain insensitivity syndromes: (a) "Got his toe caught in our gate and it ripped a big ½-inch 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." (b) "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.'" (c) "When she complains of having pain or not feeling well, it is so rare for her to do so that now we always know to listen. Another time she was trying to sit on a lunch table bench at school, missed, and hit her collar bone: they examined her at school, but since she hardly complained they let her finish out the day. When she came home, we weren't sure ourselves, but decided to have her checked out for reasons previously stated—sure enough: broken clavicle." These reports are consistent with an impairment in nociception and/or increased pain tolerance, but not complete lack of pain sensation as is observed in patients with mutations in *NGF* [9; 11; 17], *TRKA* [30] or *PRDM12* [13] genes, all of which lead to developmental loss of entire subpopulations of primary afferent nociceptors and, consequently, cause a complete loss of painful sensations [10; 19; 35]. These parental reports prompted further investigation of pain insensitivity using quantitative sensory testing.

Quantitative sensory testing.

Based on the parental reports we tested the hypothesis that *BDNF* hemizygosity may lead to an impairment in nociceptive processing. We performed QST to determine the ability of *BDNF*^{+/-} subjects to rate hot and cold stimuli. Subjects were asked to rate pain intensity of each temperature delivered using a 6-point Wong-Baker FACES Pain Rating Scale [59], which has been validated in children as young as 3 years of age; the youngest subject in our study was 6 years old (Figure 1B). The average rating of 4 replicates for each temperature (2 on the right forearm, 2 on the left forearm) was used for analyses. Figure 1D and E show the average rating for all subjects (black and red lines) and the average data for each patient (black and red circles) at each temperature. QST showed comparable ratings for non-noxious warm (35°C) and cool (29°C) temperatures ($p=0.49$ and $p=0.18$, respectively).

However, when tested over a range of hot (43–49°C) and cold (14–2°C) stimuli, *BDNF*^{+/-} WAGR subjects rated these stimuli as significantly less painful (Fig 1D, E).

Bioinformatic analysis of WAGR gene deletion locus.

Several genes are located at the deletion boundary that separates the two groups of individuals in the present study (Figure 2A), and several of these genes are expressed in pain circuit tissues (Supplementary Figure 4). Despite the large number of genes in the WAGR locus, a substantially smaller subset of genes is located in the sub-region of the locus that differentiates the two groups of WAGR subjects. Of this subset of genes, *BDNF* is the most highly enriched in pain circuit tissues such as the dorsal root ganglion (Figure 2B) as compared to other regions of the human body.

Heterozygous knockout rat.

In order to assess the role of *BDNF* haploinsufficiency specifically, we performed nociceptive testing on rats harboring a loss of function frameshift mutation within the *Bdnf* gene on one allele (*Bdnf*^{+/-}; SD-*Bdnf*^{tm1sage}, Horizon Discovery, Cambridge, UK)[54]. Expression from both alleles, and in wildtype rats, was assessed by RNA-Seq, revealing equal levels of *Bdnf* transcripts, however, approximately half of the transcript from the +/- animals contained the deletion which introduced a frameshift and stop codon shortly after the propeptide cleavage site (Supplementary Figure 3). Previous studies in the same strain have showed that serum levels of BDNF are reduced by approximately 50% [54]. All behavioral assessments in rats were performed by an individual blinded to genotype. Heat was delivered to the hind paw by a focused halogen bulb, terminating when the animal withdrew its hind limb [28; 44] (Plantar Test, Ugo Basile, Monvalle VA, Italy) and calibrated using the thermistor wire (see Methods). *Bdnf*^{+/-} rats exhibited a significantly increased latency to withdrawal corresponding to a higher temperature of withdrawal (44.6°C for +/+ vs. 46.5°C for +/-; Figure 3A, B). Sensitivity to noxious cold was tested by measuring withdrawal latency upon application of an aversive cold stimulus to the hind paw as described by Brenner *et al.* [7] using a compacted dry ice stimulus. Relative to *Bdnf*^{+/+} rats, *Bdnf*^{+/-} rats exhibited a significantly longer latency to withdraw from the cold stimulus, corresponding to a colder temperature of withdrawal (18.5°C for +/+ vs. 12.9°C for +/-; Figure 3C, D).

Heat sensitivity also was examined using the hot plate test (48°C) and measuring the latency to hind paw licking behavior [1]. The lower temperature was chosen because it is at the low end of the noxious range commonly used, where we hypothesized the greatest chance of seeing a behavioral difference between groups. *Bdnf*^{+/-} rats exhibited significantly longer latencies to lick their hind paws on the hot plate compared to the *Bdnf*^{+/+} littermates (for *Bdnf*^{+/-} compared to WT, respectively: 95.4 ± 49.3 versus 45.2 ± 10.6 sec for the first lick, and 118.2 ± 47.2 versus 68.2 ± 15.3 sec for the second lick; Figure 3E). Cold sensitivity was assessed further by recording spontaneous cold avoidance behaviors using a cold plate. Animals were tested at plate temperatures of 34°C, 23°, 10°, 8°, 6°, and 4°C (Figure 3F). From blinded evaluation of video recordings of this test we observed that, in response to noxious cold temperatures, WT rats raised their tail to avoid contact with the cold surface. Quantification of this endpoint showed that *Bdnf*^{+/-} animals exhibited significantly less

total duration of tail lifting (genotype effect, $p=0.0006$, Figure 3G), indicating a reduced, but not abolished, avoidance of noxious cold temperatures. In addition to the hot plate and C-fiber noxious heat-mediated responses, *Bdnf*^{+/-} rats were tested for responsiveness to A δ -mediated[3] hind limb withdrawal to short (100 ms) heat pulses delivered by an infrared diode laser. Animals were tested over a range of laser intensities on both the heel and plantar surfaces of the hind paw[42; 46](Figure 4). Relative to *Bdnf*^{+/+} rats, *Bdnf*^{+/-} animals showed a reduction in responsiveness to the short thermal A δ stimuli relative to WT littermates. While their responses are reduced, the rats approach 100% response rates at the highest level of intensity on the more sensitive mid-plantar area of skin (Figure 4D) indicating an ability to respond to sufficiently strong stimulation. As a non-thermal behavioral assay, both groups of rats were tested using von Frey filaments, with the *Bdnf*^{+/-} rats requiring larger filaments (more force) to evoke a withdrawal response (Supplementary Figure 5).

DRG and Dorsal Spinal Cord Transcriptomic Analyses.

Using RNA-Seq we examined gene expression in *Bdnf*^{+/-} and *Bdnf*^{+/+} rats in both DRG and dorsal spinal cord. In the DRG, our results indicated no reduction in molecular markers for any specific sensory cell type or functional modality. We previously validated this method to detect deletion vs. preservation of specific neuronal subtypes within the DRG[49]. Our quantification includes examination of genes encoding ion channels such as Trpv1 and Trpm8, which transduce hot and cold thermal stimuli (Figure 5A, Supplementary Figure 6), which were not differential in the DRG. In the dorsal horn of the spinal cord, the overall number of significantly differential genes was greater than in the DRG (Figure 5B). We did not observe alterations in known markers of spinal second order nociceptive neurons. Markers of these cells were examined because deletion of cells expressing the substance P/neurokinin1 receptor (*Tacr1*) is known to cause profound pain insensitivity[29; 39]. Of the genes that are altered, we see some evidence that neuropeptide signaling may be modified in *Bdnf*^{+/-} rats (Supplementary Figure 6, 7), with the gene encoding corticotrophin-releasing hormone (*Crh*) strongly reduced in the deleted animals in both tissues examined (Supplementary Figure 8). This is consistent with other reports where decreased *Bdnf* expression, or that of its receptor, was associated with decreased *Crh* expression[31]. However, this gene is not highly expressed, and it is unclear to what extent *Crh* signaling contributes to the *Bdnf*^{+/-} phenotype. Based on sequencing experiments of glia and other non-neural nervous system cells [62], many of the significantly differential genes are expressed in astrocytes and microglia (Figure 5C, D), pointing to changes in gene expression programs within glial cells, perhaps responding to, or driven by altered neuronal signaling in the spinal cord (Supplementary Figure 9). Ingenuity Pathway Analysis was performed to identify common hub genes based on the observed gene changes. Based on these analyses, many of the genes observed to be altered in *Bdnf*^{+/-} animals are related to interferon signaling, and all of them are upregulated (Figure 5E, Supplementary Figure 10).

Discussion

The majority of human genetic pain insensitivity syndromes described to date affect primary afferent neurons, leading to inability to transduce noxious stimuli[50]. The present study

supports the finding that *Bdnf* haploinsufficiency leads to modulation of pain sensitivity without loss of primary afferent neurons, a result consistent with previous studies showing unaltered DRG nociceptor populations in homozygous *Bdnf* knockout mice[26; 63]. Behavioral results from thermal testing of both human WAGR subjects and *Bdnf*^{+/-} rats point to a model where *BDNF* haploinsufficiency produces an elevated pain threshold that can be superseded by sufficiently strong stimulation. Our transcriptomic evidence points to a broader alteration in gene expression from *Bdnf* haploinsufficiency in dorsal spinal cord than in DRG (Figure 5). In concert with our behavioral battery, this suggests a system where transmission in peripheral afferent nociceptors is intact, but the synaptic transmission of these signals to spinal second order neurons, or transmission of the signals by second order neurons to higher CNS regions, is potentially disrupted, requiring abnormally strong stimuli to elicit responses[40]. In part, this may be attributable to altered synaptic efficacy between components of the nociceptive circuitry, which has been reported at a number of synapses throughout the CNS[5; 52]. Given the lack of gene signatures indicative of DRG neuronal disruption, the results are suggestive of congenital indifference to pain[45], although parsing indifference vs. insensitivity in WAGR subjects is challenging due to their rarity, young age, and intellectual disabilities.

Previous experiments using *Bdnf* knockout mice to examine basal pain sensitivity have yielded conflicting results, with at least one study showing sensitization to some forms of thermal stimulation in DRG-specific *Bdnf*^{-/-} mice[63]. At least one other study using *Bdnf*^{+/-} mice showed no difference in baseline thermal pain sensitivity[60]. To add to these apparently conflicting results, *reduced* sensitivity to thermal sensations has been observed using a hotplate test in both *Bdnf*^{+/-} mice, and in DRG-specific *Bdnf*^{-/-} mice[36; 51] consistent with our results in the rat. Overall, strain and species differences as well as technical elements may have contributed to some differences in findings. However, based on mouse studies, it has generally been accepted that BDNF plays a role in the transition from acute to chronic pain, and not in the determination of pain sensitivity[51]. The *Bdnf* gene is strongly upregulated by persistent inflammatory pain[38](Supplementary Figure 11), supporting its role in the plasticities that occur in the transition between acute and chronic pain[47]. Our results point to actions of *Bdnf* in spinal cord or other pain modulatory circuit regions, such as the brainstem[47] and are consistent with earlier studies in rat which indicated a CNS site of action[32]. It has also been suggested that spinal BDNF contributes to hyperalgesia by sensitizing second order dorsal horn neurons[4; 20]. BDNF secretion from glial cells has been implicated in regulating many functions related to learning and memory[53], and has been implicated in modulation of pain states[14]. Based on these aggregated findings, BDNF most likely act at a variety of different sites throughout the neuraxis to modulate pain sensitivity.

However, it seems likely that BDNF does modulate the activity of glial cells in the spinal cord, based on the relatively large number of regulated genes in the spinal cord that are differentially expressed in response to *Bdnf* haploinsufficiency. Within this gene set, the most prominent changes are related to interferon signaling, which is known to participate in neuron-glia interactions, and which has been shown to regulate pain states[55]. In addition to this, it is notable that two of the most highly regulated, most highly expressed genes in our dataset (*Serpina3n* and *Fam111a*) are the only two genes identified by a microarray-

based study examining high and low pain-sensitive animals after a sciatic nerve ligation model of neuropathic pain[57]. Of particular interest, Serpina3n, a secreted serine protease inhibitor, was posited by the authors of that study to participate in the intercellular communication between neurons and immune cells. Relatedly, treatment of leukocytes with Serpina3n prevented neuronal killing in a neurodegeneration context[22]. In the context of the present paper it is perhaps more likely that the biological process engaged has to do with the formation of appropriate synapses and/or removal or pruning of synapses, as this process is known to involve Bdnf, neuron-glia interactions, and interferon signaling[2].

The significance of *BDNF* as a pain modulatory gene is evident based on the magnitude and penetrance of the observed reduction in pain sensitivity phenotype. Pain drives individuals to seek medical intervention. In the specific case of WAGR syndrome, these patients are at risk for developing pancreatitis[18], which is frequently accompanied by severe abdominal pain. Diagnosis and treatment of painful medical emergencies can be delayed in patients with pain insensitivity[37] such as that observed in WAGR syndrome, emphasizing a need for close monitoring of patient symptoms. In terms of translational potential for analgesia, our data suggest that *BDNF* may be part of a larger gene network that modulates the complex trait of pain sensitivity in the general population[50]. Additional studies may further elucidate the relationship between *BDNF* and nociceptive sensitivity, a topic which has been investigated in a limited manner with regard to the relatively common Val66Met polymorphism[15; 58]. Additionally, while this study did not specifically examine a potential role of proBDNF in pain, recent work has investigated its role in hippocampal plasticity[21; 43]. Ultimately, a greater understanding of these pain regulatory circuits may aid in the development of new analgesic approaches.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This research was supported by the Intramural Research Program of NICHD and the NIH Clinical Center with supplemental funding from the NIH Bench-to-Bedside Program to J.C.H. and to M.J.I. from NCCIH and the Office of Behavioral and Social Sciences. We thank Matthew Tsang, Miriya Tune, Kyra Jefferson-George, and Jamila Crossman for technical assistance. We also thank the families of the International WAGR Syndrome Association for promotion and participation in the clinical studies. The authors thank Xiaobai Li for assistance with statistical analyses.

Abbreviations

WAGR	Wilms tumor, Aniridia, Genitorinary anomalies, and mental Retardation
BDNF	Brain-derived neurotrophic factor
QST	Quantitative sensory testing

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Figure 1. Characteristics and thermal pain perception ratings of patients with WAGR syndrome. A cohort of 12 WAGR syndrome subjects were tested for thermal pain ratings.

This cohort consisted of 6 *BDNF*^{+/-} and 6 *BDNF*^{+/+} subjects with normal nerve conduction measurements (Supplementary Tables 1, 2), selected from a larger cohort of 32 patients with WAGR syndrome [23] based on capacity to understand and perform behavioral pain ratings. (A) 11p deletion boundaries, determined as previously described [23] are shown for each subject (N=6) with the deleted region indicated in black. These deletions can be inclusive or exclusive of the *BDNF* gene (shown in red), allowing us to divide the subjects by *BDNF* genotype. (B) *BDNF*^{+/-} versus *BDNF*^{+/+} subjects were similar in age, sex distribution, adaptive behavior (Vineland), cognitive functioning (IQ), assessed as previously described [24], and history of vincristine treatment for Wilms tumor. (C) Parents of *BDNF*^{+/-} individuals reported significantly more examples of pain insensitivity in their children in the optional comments section of a questionnaire about pain sensitivity. (D,E) Thermal pain perception was tested for hot (D) and cold (E) temperatures by application of a 1.6 × 1.6 cm Medoc thermode to the volar forearm followed by patient reports of pain intensity using a 6-point Wong-Baker FACES Pain Rating Scale [59]. Baseline measurements were also taken for non-noxious warm (35°C) and cool (29°C) stimuli. These measurements were used to create linear mixed-effects models for hot and cold pain ratings, using baseline rating and IQ as covariates in the model. For both hot and cold temperature ratings, genotype showed a significant effect, indicating that *BDNF*^{+/+} and *BDNF*^{+/-}

individuals rated both heat and cold stimuli as less painful (D, E). For hot temperatures, there was a significant effect of temperature on rating (D).

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Figure 2. Enrichment analysis of selected WAGR locus genes compared to 53 human tissues in the GTEx database.

(A,B) Many of the genes deleted in patients in the present study are expressed in pain circuit tissues in humans. (A) We examined the subset of genes within the deletion boundaries shared by the *BDNF*^{+/-} individuals but not the *BDNF*^{+/+} individuals. (B) To address whether these genes are enriched in these tissues, expression values (sFPKM, MAGIC) were compared with reported median RPKM gene expression values in the GTEx database v6, and ordered by degree of enrichment. The top 20 human tissues enriched for these genes are shown out of a total of 55 tissues examined. Of the genes within this locus, *BDNF* is the most highly enriched gene in human dorsal root ganglia and dorsal spinal cord samples relative to other tissues. It shows expression in other neural tissues, such as the cerebellum. Notably, the *ANO3* gene, which is also among the most enriched genes has been implicated in nociceptive sensitivity in rats, where knockout studies of the orthologous rat gene caused increased thermal and mechanical pain sensitivity[27]. These alterations in pain sensitivity have not been reported in humans with *ANO3* mutations to date, who present with cervical dystonia[12].

Figure 3. Thermal pain testing in *Bdnf*^{+/-} rats.

Behavioral assessment of thermal nociception was performed in rats by application of thermal stimuli to the plantar surface of the hind paw. (A) Radiant heat stimulation was applied and behavior was rated as withdrawal latency *Bdnf*^{+/-} rats (N=5) withdrew significantly later than WT animals. (B) A thermistor probe was placed under the paw of awake behaving *Bdnf*^{+/-} and WT rats to measure the temperature ramp over time occurring at the dermal surface (N=21). Withdrawal latency data is superimposed as gray (WT) or red (*Bdnf*^{+/-}) bars showing that WT animals withdraw at approximately 44.8°C, while *Bdnf*^{+/-} animals withdraw at approximately 46.5°C. (C) Noxious cold stimulation was performed by indirect contact with dry ice. *Bdnf*^{+/-} animals (N=10), withdrew later in response to noxious cold than WT (N=10) (D) Behavioral response was elicited at approximately 20.0°C plantar

dermal temperature in WT, and at approximately 14.0°C for *Bdnf*^{+/-} rats. (E) When placed on a 48°C hot plate both groups responded by licking their hind paw. In both groups the first lick was followed by a second lick, which was also quantified (E; N=5 for each group). The latency to both the first and second hind paw lick was longer in *Bdnf*^{+/-} rats. (F) When placed on a cold plate, both groups of animals displayed avoidance behavior to the cold surface evidenced by raising the tail to reduce contact area, with WT exhibiting significantly more tail lifting than *Bdnf*^{+/-} animals (N=5; Genotype effect $p = 0.0006$). An example of an animal lifting his tail in response to noxious cold is shown (G). (A) Two-tailed Student's T-test; C, Two-tailed Mann-Whitney test; (E) Fisher's Exact test; (F) linear mixed-effects model. Error bars represent SEM; *, $p < 0.05$; **, $p < 0.01$.

Figure 4. Short-pulse A-δ laser stimulation of WT and *Bdnf*^{+/-} heel and plantar surface of the hind paw.

A 100ms pulse was delivered to the heel (A, C, E) or plantar surface (B, D, F) across a range of stimulus intensities, and behavioral assessments were scored by a blinded observer (N=5). Ratings of behavioral responses were made using a previously validated scale [41; 42], with significant differences observed on the plantar (B), but not the heel (A). The plantar area is more sensitive to thermal stimulation than the heel, resulting in lower overall responsiveness to the same stimulus intensity on the heel (C,D). Response ratios to hind paw stimulation were significantly different ($P < 0.01$) for both the heel and plantar stimulation paradigms. Differences in average area under the curve (AUC) measurements were calculated using a Wilcoxon Mann-Whitney U-test; *, $p < 0.05$; **, $p < 0.01$.

Figure 5. RNA-Seq analysis of dorsal root ganglia and dorsal spinal cord samples from *Bdnf*^{+/-} and *Bdnf*^{+/+} rats.

DRG (N=5) and dorsal spinal cord (N= 10) were collected from *Bdnf*^{+/-} and *Bdnf*^{+/+} rats and RNA-Seq was performed. Significantly differentially-expressed genes between these two groups were plotted according to level of expression (average sFPKM) and fold difference (mutant/WT). The dotted line at 1 sFPKM highlights genes which are highly expressed. Compared to (A) DRG, a larger number of genes are differentially expressed in (B) the dorsal horn and these genes are also more highly differential. (C) GO analysis was performed, with one term “defense response to virus” significantly enriched, suggesting the

involvement of non-neural immune cells. (D) Highly expressed significantly differential genes were queried against a database sampling mouse cerebral cortex cell populations [62], showing that most of the differential genes are expressed in glial and other supportive non-neural cells. (E) A large percent of the most upregulated genes are involved in interferon signaling. This relationship was also examined further by IPA analysis in Supplementary Figure 9.