

# GTP cyclohydrolase and tetrahydrobiopterin regulate pain sensitivity and persistence

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**We report that GTP cyclohydrolase (GCH1), the rate-limiting enzyme for tetrahydrobiopterin (BH4) synthesis, is a key modulator of peripheral neuropathic and inflammatory pain. BH4 is an essential cofactor for catecholamine, serotonin and nitric oxide production. After axonal injury, concentrations of BH4 rose in primary sensory neurons, owing to upregulation of GCH1. After peripheral inflammation, BH4 also increased in dorsal root ganglia (DRGs), owing to enhanced GCH1 enzyme activity. Inhibiting this *de novo* BH4 synthesis in rats attenuated neuropathic and inflammatory pain and prevented nerve injury-evoked excess nitric oxide production in the DRG, whereas administering BH4 intrathecally exacerbated pain. In humans, a haplotype of the *GCH1* gene (population frequency 15.4%) was significantly associated with less pain following diskectomy for persistent radicular low back pain. Healthy individuals homozygous for this haplotype exhibited reduced experimental pain sensitivity, and forskolin-stimulated immortalized leukocytes from haplotype carriers upregulated GCH1 less than did controls. BH4 is therefore an intrinsic regulator of pain sensitivity and chronicity, and the GTP cyclohydrolase haplotype is a marker for these traits.**

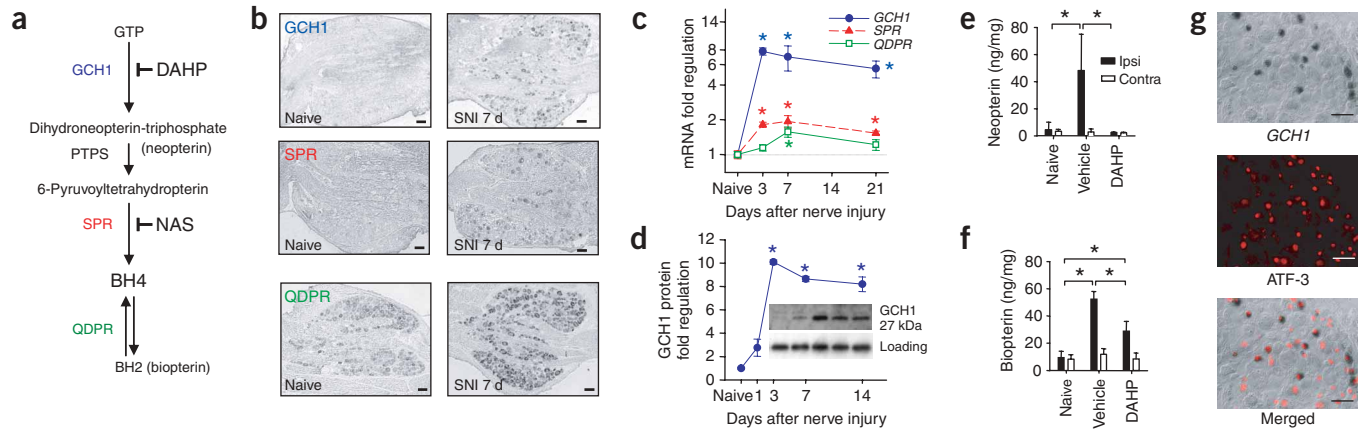
Inflammatory and neuropathic pain result from multiple changes in the peripheral and central nervous systems. Among these are increased excitability and reduced thresholds of primary sensory neurons<sup>1</sup>, altered spinal cord synaptic processing<sup>2</sup>, loss of inhibitory interneurons<sup>3</sup> and modifications of brainstem input to the spinal cord<sup>4</sup>. These changes in neuronal activity result from *de novo* gene transcription, post-translational modifications<sup>5</sup>, alterations in ion channel conductivity<sup>6</sup> and receptor function<sup>7</sup>, neuroimmune interactions<sup>8</sup> and neuronal apoptosis<sup>9</sup>. Hypersensitivity, manifesting as spontaneous pain, pain in response to normally innocuous stimuli (allodynia) and an exaggerated response to noxious stimuli (hyperalgesia) are the dominant features of clinical pain, and in some individuals they persist long after the initial injury is resolved. It is not understood what perpetuates pain hypersensitivity in only a subset of people. Inbred rodent strain and human twin studies indicate that the risk of developing chronic pain may be genetically determined<sup>10–12</sup>.

To reveal genes involved in producing persistent neuropathic pain, we searched the several hundred genes regulated in the dorsal root ganglion (DRG) following sciatic nerve injury<sup>13</sup> for those belonging to common metabolic, signaling or biosynthetic pathways and identified upregulation of two of the three enzymes in the synthesis cascade of 6(R)-L-erythro-5,6,7,8-tetrahydrobiopterin (BH4). The regulated enzymes are GTP cyclohydrolase, which catalyzes the first, rate-limiting step, and sepiapterin reductase, which performs the final conversion of 6-pyruvoyltetrahydropterin to BH4 (Fig. 1a).

BH4 is an essential cofactor for phenylalanine, tyrosine and tryptophan hydroxylases and for nitric oxide synthases. BH4 availability is critical, therefore, for catecholamine, serotonin and nitric oxide synthesis and for phenylalanine metabolism<sup>14</sup>. Production of BH4 is regulated by GTP cyclohydrolase transcription and activity<sup>15,16</sup>. Phosphorylation<sup>17</sup>, feed-forward activation through phenylalanine<sup>18</sup> and

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**Figure 1** Regulation of BH4-producing enzymes in the DRG after nerve injury. **(a)** Biosynthetic pathway of BH4. PTPS, pyruvoyltetrahydropterin synthase. **(b,c)** Upregulation of BH4-producing enzymes in L4-5 DRG neurons in the SNI model of peripheral neuropathic pain as detected by **(b)** *in situ* hybridization 7 d after SNI (**b**; scale bar 100 μm) and by **(c)** QRT-PCR (**c**;  $n = 4$ , error s.e.m.). ANOVA was consistent with differential expression of GTP cyclohydrolase (*GCH1*), sepiapterin reductase (*SPR*) and quinoid dihydropteridine reductase (*QDPR*) at the indicated time points ( $*P < 0.05$ ). **(d)** GCH1 protein expression in L4-5 DRGs after SNI ( $n = 3$ , error s.e.m.). **(e,f)** Neopterin (**e**) and biopterin (**f**) concentrations (ng per mg protein) in ipsi- and contralateral L4-5 DRGs 7 d after SNI. DAHP administered 3 h before tissue dissection reduced neopterin and biopterin ( $n = 6$ , error s.e.m.). **(g)** Three days after SNI, *GCH1* mRNA-positive neurons are also immunoreactive for the injury-induced transcription factor ATF-3 (scale bar 20 μm).  $*P < 0.05$ .

feedback inhibition through BH4 tightly regulate GTP cyclohydrolase activity. Mutations in GTP cyclohydrolase or sepiapterin reductase that cause monoamine neurotransmitter deficiency result in DOPA-responsive motor, psychiatric and cognitive disorders<sup>19,20</sup>. Given the vital roles of these neurotransmitters, increasing BH4 concentrations may profoundly impact neuronal signaling. We now show that BH4 concentrations are critical for neuropathic and inflammatory pain and that a genetic polymorphism of GTP cyclohydrolase is associated with reduced pain sensitivity and chronicity in humans, owing to reduced BH4 production.

## RESULTS

### Upregulation of BH4-producing enzymes

We studied the expression of GTP cyclohydrolase, sepiapterin reductase and quinoid dihydropteridine reductase over time in the fourth and fifth lumbar (L4-5) DRGs in the spared nerve injury<sup>21</sup> (SNI) model of peripheral neuropathic pain in rats. This model produces long-lasting pain hypersensitivity, including mechanical and cold allodynia. Transcripts of all these enzymes were upregulated (**Fig. 1b** *in situ*, **Fig. 1c** quantitative RT-PCR (QRT-PCR)), with a more than sixfold sustained increase in GTP cyclohydrolase and more modest increases of the other two. Upregulation of GTP cyclohydrolase mRNA was accompanied by increased protein expression (**Fig. 1d**) and activity (**Fig. 1e**), the latter indicated by elevated neopterin, an inactive metabolite of dihydroneopterin triphosphate (an intermediate product in the synthesis cascade)<sup>22</sup>. SNI caused a large elevation of the end product, BH4, as indicated by increases in its stable oxidation product biopterin (**Fig. 1f**). Double labeling of GTP cyclohydrolase mRNA and the injury-induced nuclear transcription factor ATF-3 (ref. 23) showed that  $97 \pm 3\%$  of neurons upregulating GTP cyclohydrolase were ATF-3-positive (**Fig. 1g**). Seven days after SNI,  $65 \pm 13\%$  of L5 DRG neuronal nuclei expressed ATF-3, reflecting the proportion of cells with axonal damage<sup>21</sup>. Of these,  $75 \pm 4\%$  upregulated GTP cyclohydrolase mRNA.

### Inhibiting GTP cyclohydrolase reduces neuropathic pain

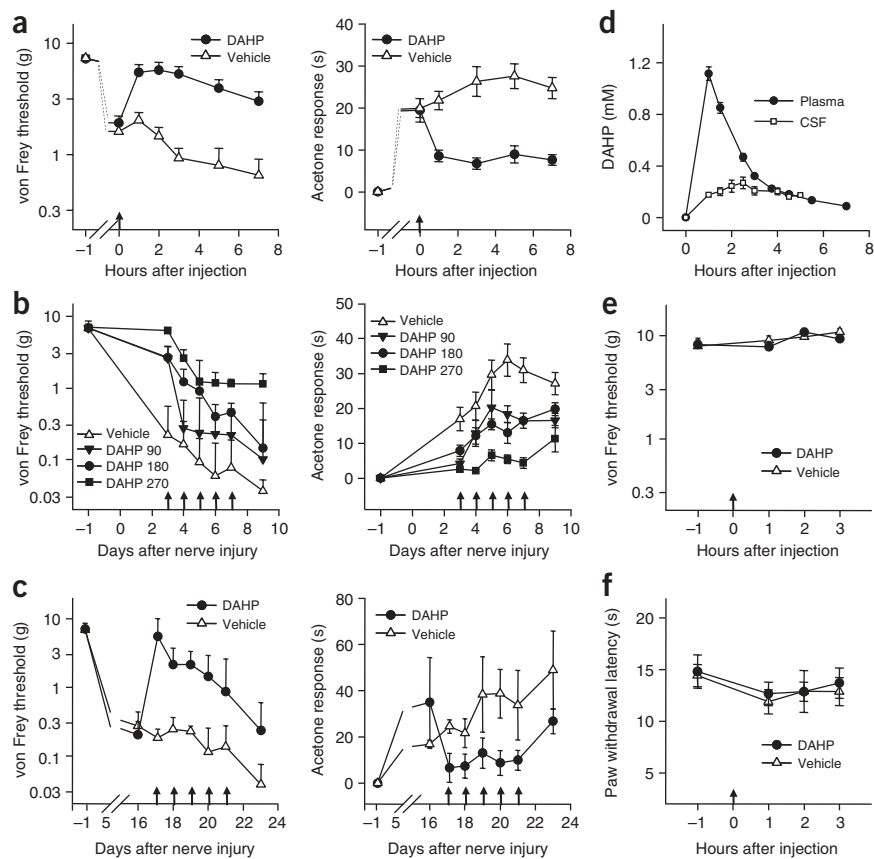
To test whether increased BH4 synthesis contributes to neuropathic pain, we analyzed the effects of the prototypic GTP cyclohydrolase

inhibitor 2,4-diamino-6-hydroxypyrimidine<sup>24,25</sup> (DAHP) in the SNI model.

Injection of a single dose of DAHP (180 mg per kg body weight intraperitoneally (i.p.)) four days after SNI reversed mechanical and cold pain hypersensitivity within 60 min (**Fig. 2a**). DAHP produced dose-dependent antinociceptive effects on repeated administration without loss of activity (**Fig. 2b**). It had similar antinociceptive effects when first administered seventeen days after SNI surgery (**Fig. 2c**), produced analgesia in the chronic constriction injury (CCI) and spinal nerve ligation (SNL) models of peripheral neuropathic pain (**Supplementary Fig. 1** online) and was effective on spinal delivery (250 μg/kg/h) at 1/30<sup>th</sup> of the systemic dose (**Supplementary Fig. 2** online). The antinociceptive effect of DAHP paralleled the time course of its plasma and CSF concentrations (**Fig. 2d**), which were within the IC<sub>50</sub> range (100–300 μM) for GTP cyclohydrolase inhibition *in vitro*<sup>24,25</sup>. DAHP treatment at this dose prevented nerve injury-induced increases in neopterin in the DRG (**Fig. 1e**), and it significantly reduced biopterin concentrations (**Fig. 1f**). Biopterin concentrations did not return to preinjury baselines because recycling of BH4 from its oxidation products is not inhibited by DAHP. DAHP (180 mg/kg i.p.) did not change mechanical or heat pain sensitivity in naive animals (**Fig. 2e,f**) and had no effect on body weight, activity, or performance in the forced swim test<sup>26</sup> (**Supplementary Fig. 2**).

### Blocking GTP cyclohydrolase inhibits inflammatory pain

Inflammation produced by hindpaw injection of complete Freund adjuvant (CFA) did not increase GTP cyclohydrolase mRNA expression in the DRG (**Supplementary Fig. 3** online). However, intraplantar CFA injection caused significant increases in GTP cyclohydrolase enzyme activity, with increases of neopterin (**Fig. 3a**) and biopterin (**Fig. 3b**) in L4-5 DRGs. DAHP (180 mg/kg i.p.) reduced heat hyperalgesia in the inflamed hindpaw (**Fig. 3c,d**), when administered both before (**Fig. 3c**) and 24 h after intraplantar (hind paw) CFA injection (**Fig. 3d**), and normalized neopterin and biopterin concentrations in the DRGs (**Fig. 3a,b**). Similar efficacy was achieved with intrathecal DAHP (**Supplementary Fig. 2**; 1/30<sup>th</sup> systemic dose). DAHP (180 mg/kg i.p.) also reduced flinching behavior in the first



**Figure 2** Efficacy of DAHP in the spared nerve injury model of neuropathic pain. **(a)** Injection of DAHP 4 d after SNI (arrow) significantly reduced mechanical (von Frey) and cold (acetone) allodynia ( $n = 12$ ,  $P < 0.05$ ). **(b)** Dose dependent efficacy of DAHP (90, 180 and 270 mg/kg) on mechanical and cold allodynia with repeated daily injections (arrows) in the SNI model, measured 2–3 h after injections ( $n = 9–10$ ,  $P < 0.05$ ). The relationship between dose and effect was linear ( $R = 0.709$  and  $R = 0.754$  for mechanical and cold allodynia respectively,  $P < 0.001$ ). **(c)** DAHP treatment starting 17 d after nerve injury produced a significant reduction of mechanical and cold pain hypersensitivity ( $n = 7$ ,  $P < 0.05$ ). **(d)** DAHP plasma and CSF concentration time courses after i.p. injection. **(e,f)** DAHP treatment (arrow) failed to modify mechanical and thermal threshold in naive animals ( $n = 6$ ,  $P = 1$ ). For all figures error bars represent s.e.m. The areas under the effect-versus-time curves were used for statistical comparisons of drug effects.

pain-producing effects may be mediated through excess activity of these enzymes. After SNI, we found upregulation of the genes encoding neuronal tryptophan hydroxylase and neuronal nitric oxide synthase (nNOS) in ipsilateral DRGs (**Fig. 5a**), but no change in those encoding phenylalanine hydroxylase or endothelial or inducible NOS, and a downregulation of the gene encoding tyrosine hydroxylase (**Fig. 5b**). Despite upregulation of the gene encoding neuronal tryptophan hydroxylase in the DRG, serotonin concentrations in DRGs from naive and SNI animals were below limits of quantification (50 pg/ml; data not shown). Upregulation of the gene encoding nNOS was accompanied by an increase in nitric oxide concentrations in the L4–5 DRGs at 7 d (**Fig. 5c**) that was prevented by DAHP treatment. The NOS inhibitor L-NAME (25 mg/kg i.p.) reduced SNI-evoked mechanical and cold allodynia tested 4 d after SNI (**Fig. 5d**). Antinociceptive effects of DAHP may be mediated at least in part, therefore, by prevention of excess NO production.

To further analyze potential mechanisms, we employed calcium imaging with cultured adult rat DRG neurons. BH4 (0.3–10  $\mu$ M) dose-dependently increased intracellular calcium concentrations in 67% of recorded cells ( $n = 95$ ; **Fig. 5e**). BH4 elevated calcium within seconds, and this was abolished by a calcium-free perfusate, indicating increased calcium influx ( $n = 12$ ). The NO-releasing substance diethylamine NONOate diethylammonium salt (DEA-NONOate; 50  $\mu$ M) produced similar increases in intracellular calcium concentration ( $[Ca^{2+}]_i$ ), which were also mediated by calcium influx ( $n = 32$ ). The NOS inhibitor *N* $\omega$ -nitro-L-arginine methyl ester hydrochloride (L-NAME; 50  $\mu$ M) reduced the BH4 effect by  $47 \pm 4\%$  ( $n = 29$ ,  $P < 0.01$ ; **Fig. 5f**) suggesting that BH4 acts partly, but not exclusively, through NOS.

Bath application of BH4 to an isolated adult rat spinal cord slice did not change the frequency or amplitude of AMPA receptor-mediated miniature excitatory postsynaptic currents or produce direct inward currents in superficial dorsal horn neurons (BH4 10  $\mu$ M,  $n = 6$ ; 20  $\mu$ M,  $n = 2$ ; data not shown), indicating that BH4, in contrast to nitric oxide<sup>28</sup>, does not increase glutamatergic transmission.

and second phases of the formalin test, indicators of acute nociception and activity-dependent central sensitization (**Fig. 3e**), and this was associated with a reduction in c-FOS-immunoreactive neurons in the dorsal horn of the spinal cord (**Fig. 3f,g**).

### Blocking sepiapterin reductase inhibits pain

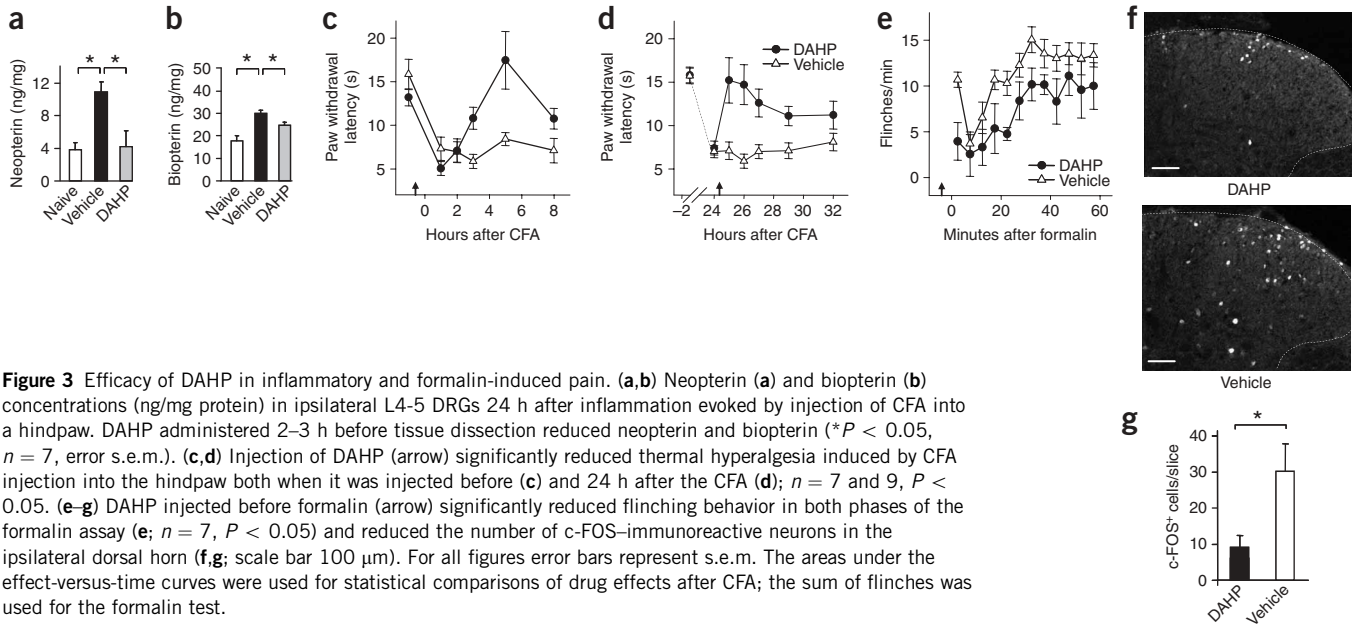
To substantiate that the analgesic effects of DAHP resulted from reduced BH4 synthesis, we also tested the effect of *N*-acetylserotonin (NAS), an inhibitor of sepiapterin reductase<sup>27</sup>. NAS (100  $\mu$ g/kg/h i.t. for 14 d) significantly reduced mechanical and cold allodynia after SNI (**Fig. 4a,b**) without overt adverse effects. Intraperitoneal injection of a single dose of NAS (50 mg/kg i.p.) before intraplantar CFA injection reduced thermal hyperalgesia in this paw inflammation model (**Fig. 4c**). NAS also reduced total biopterin concentrations in L4–5 DRGs after SNI, indicating inhibition of BH4 synthesis (**Fig. 4d**).

### Tetrahydrobiopterin induces pain hypersensitivity

To determine if BH4 enhances pain sensitivity we injected its active enantiomer 6(*R*)-5,6,7,8-tetrahydrobiopterin dihydrochloride intrathecally (i.t.) via a lumbar spinal catheter (1  $\mu$ g/ $\mu$ l, 10  $\mu$ l). BH4 caused a rapid and long-lasting increase in the response to noxious radiant heat in naive rats (**Fig. 4e**). Intrathecally injected BH4 also further increased pain sensitivity in both the SNI (**Fig. 4f**) and the CFA models of neuropathic and inflammatory pain, respectively (**Fig. 4g**). The inactive metabolite neopterin (1  $\mu$ g/ $\mu$ l, 10  $\mu$ l i.t.) had no effect (**Fig. 4h**).

### Potential mechanisms

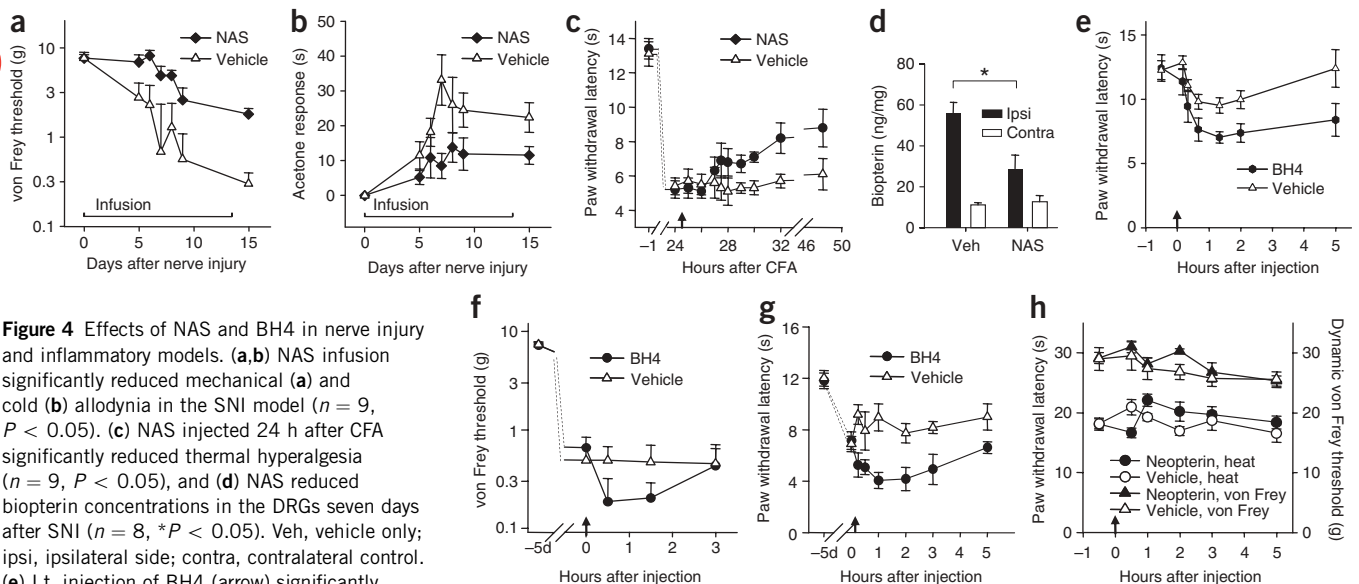
Availability of BH4 regulates activity of nitric oxide synthases as well as of tyrosine and tryptophan hydroxylases. Therefore, its

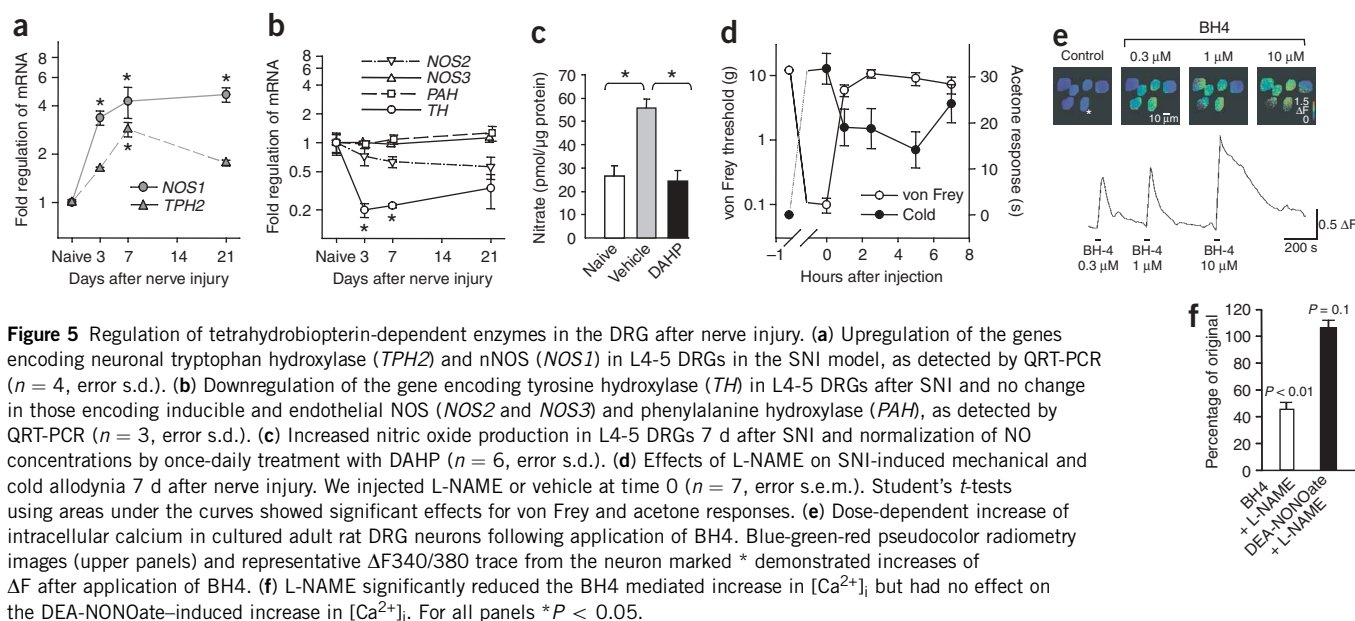


### Pain-protective haplotype of GTP cyclohydrolase

We hypothesized that polymorphisms in the human gene encoding GTP cyclohydrolase (*GCH1*) might be associated with a distinct pain phenotype. We genotyped 168 Caucasian adults who participated in a prospective observational study of surgical discectomy for persistent lumbar root pain caused by intervertebral disc herniation<sup>29,30</sup> (demographic data, **Supplementary Table 1** online) for 15 single nucleotide polymorphisms (SNPs) spaced evenly in *GCH1* (**Fig. 6a**). Five SNPs in *GCH1* (**Fig. 6a**, **Supplementary Table 2** online) were significantly associated with scores of persistent leg pain over the first postoperative year, prespecified as the primary outcome. *GCH1* had a

single conserved haplotype block 72 kb in size (**Supplementary Fig. 4** online). Stepwise regression analysis<sup>31</sup> identified one *GCH1* haplotype, with an allelic frequency of 15.4%, as highly associated with low scores for persistent leg pain ( $P = 0.009$ ). **Figure 6b** shows representative raw pain scores over time for the frequency of leg pain at rest, one of four variables used to calculate the pain z-score. In the 147 subjects who completed the 1-year questionnaire, the numbers of those who reported that their leg pain was worse, unchanged or only a little better were 0/4 of those with two copies of the pain-protective haplotype (mean pain z-score 0.06), 4/41 of those with one copy (mean pain z-score 0.44) and 22/102 of those with no copies (mean pain z-score 0.80).





Comparison of the haplotypes shows that the two uncommon alleles (A and T) of the two SNPs (C.-9610>A and C.343+8900A>T) significantly associated with low pain score are unique to the pain-protective haplotype (Fig. 6a).

We next explored whether this pain-protective haplotype was also associated with reduced heat, ischemic and pressure pain sensitivity in two cohorts of healthy volunteers (Supplementary Table 4 online). Individuals carrying two copies of the pain-protective haplotype were significantly less sensitive to mechanical pain and tended to be less sensitive to heat and ischemic pain (Fig. 6c).

We analyzed *GCH1* mRNA and protein expression and BH4 synthesis in Epstein-Barr virus (EBV)-immortalized leukocytes of subjects who participated in the lumbar root pain study<sup>29,30</sup>. Baseline expression (mRNA and protein) of *GCH1* and BH4 concentrations did not significantly differ between carriers and noncarriers of the haplotype. Since *GCH1* transcription increases in response to cyclic AMP, acting through regulatory elements located in the proximal promoter<sup>32,33</sup>, we stimulated the cells with forskolin (10  $\mu$ M, 12 h) to increase adenylyl cyclase activity. Forskolin increased *GCH1* mRNA (Fig. 6d), *GCH1* protein (Fig. 6e) and BH4 production (Fig. 6f) in white blood cells (WBCs) from individuals with no copies of the pain-protective haplotype. The upregulation by forskolin of the *GCH1* transcript was significantly smaller in leukocytes from individuals with one or two copies of the pain-protective haplotype (Fig. 6d). In contrast to those in noncarriers, *GCH1* protein levels in WBCs (Fig. 6e) and biopterin concentrations in WBC culture supernatants (Fig. 6f) fell below baseline in homozygous haplotype carriers, indicating that the haplotype may modify protein stability. Cells from heterozygous carriers had an intermediate phenotype (Fig. 6e,f). We further analyzed biopterin in whole blood of healthy homozygous 0/0 and X/X volunteers. Baseline biopterin concentrations were slightly higher in homozygous carriers of the haplotype compared with noncarriers (data not shown). Following forskolin (10  $\mu$ M, 24 h), biopterin increased by about 60% in noncarriers, compared with 20% in homozygous carriers of the haplotype (Fig. 6g). Differences between WBCs and whole blood may be caused by BH4 recycling by quinoid dihydropteridine reductase in erythrocytes.

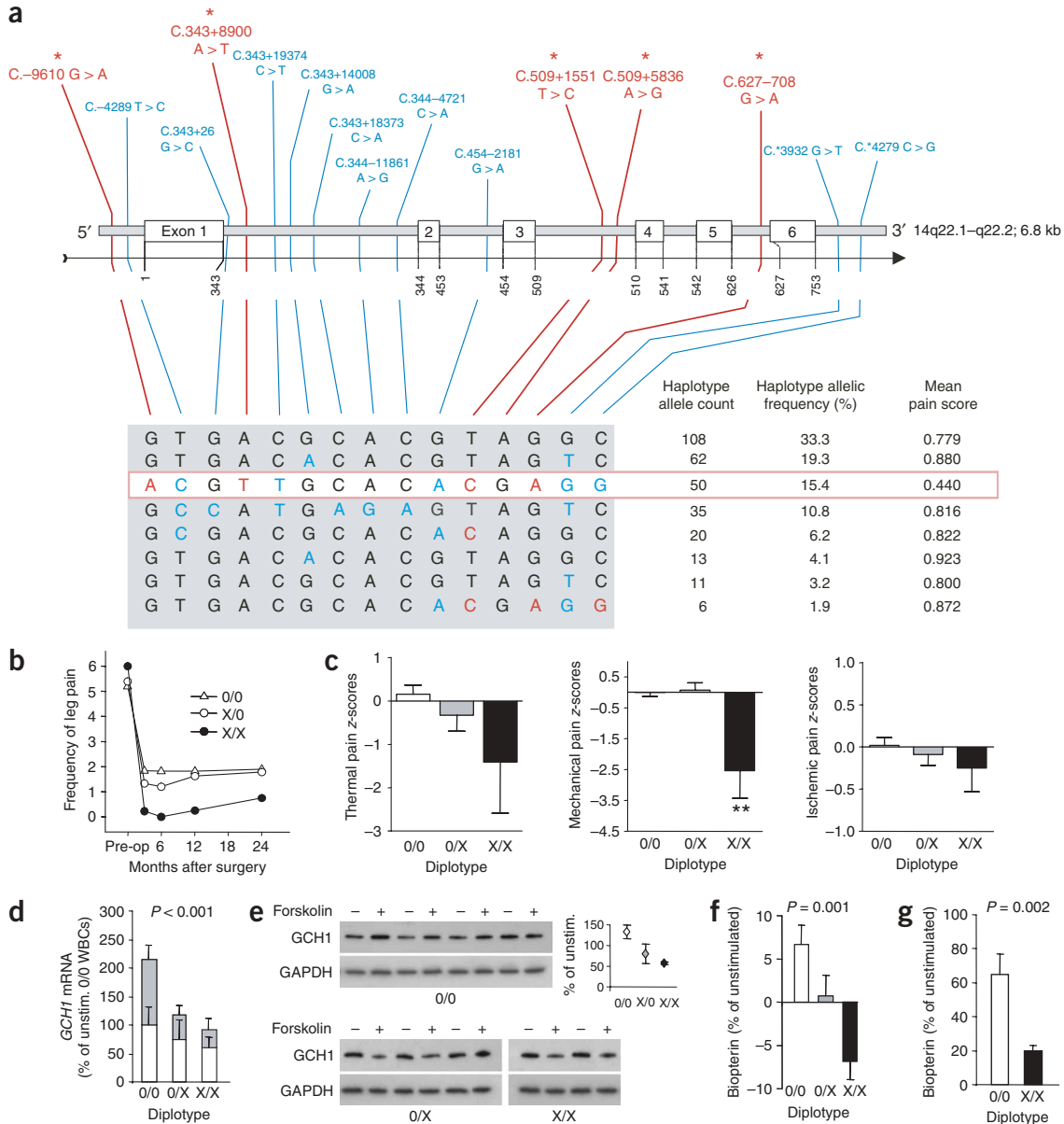
## DISCUSSION

Tetrahydrobiopterin synthesis increased in rat sensory neurons in response to both axonal injury and peripheral inflammation. Blocking the increased BH4 synthesis by independently inhibiting two successive enzymes in the synthesis cascade reduced neuropathic and inflammatory pain, and in contrast, BH4 administration produced pain in naive animals and enhanced inflammatory and neuropathic pain sensitivity. Furthermore, a haplotype of *GCH1* that reduces its upregulation in response to a forskolin challenge was protective against persistent neuropathic pain and was associated with reduced sensitivity to experimental pain in humans. We conclude that we have identified both a previously unknown pathway involved in the production and modulation of pain and a genetic marker of pain sensitivity.

Regulation of BH4 concentrations is normally achieved by a feedback inhibition mediated by BH4 acting in concert with the GTP cyclohydrolase feedback regulatory protein (GFRP). GFRP, however, unlike GTP cyclohydrolase, is not upregulated after nerve injury (QRT-PCR data not shown). This altered stoichiometry may disrupt efficient feedback inhibition, resulting in accumulation of excess BH4 in DRG neurons, and thereby increasing nitric oxide and possibly biogenic amine neurotransmitter synthesis. Seven days after SNI, nitric oxide concentrations were elevated in the DRG, suggesting that overproduction of NO contributes to the pain evoked by BH4. Pain-producing effects of nitric oxide probably involve direct nitrosylation of target proteins<sup>34</sup>, modulation of NMDA receptor activity<sup>35</sup> and activation of the guanylyl cyclase-cyclic GMP-PKG pathway<sup>36,37</sup>, resulting in increased glutamatergic transmission<sup>38</sup>. Supporting this, inhibition of GTP cyclohydrolase prevented the increases in both BH4 and NO, and NOS inhibition reduced mechanical and cold allodynia after SNI. BH4 may act in a paracrine as well as an autocrine fashion, as it is released from neurons<sup>39</sup> and may both increase enzymatic activity and produced cofactor-independent effects<sup>40,41</sup>. Considering the latter, we found that BH4 produced a short-latency calcium influx in cultured adult DRG neurons that is partly mediated through nitric oxide synthesis. Although neuronal tryptophan hydroxylase mRNA was upregulated in DRG neurons after SNI, serotonin concentrations remained below detection limits in this tissue. In the spinal cord

serotonin is expressed in descending inhibitory and excitatory fibers. DAHP treatment did not, however, significantly reduce serotonin concentrations in the spinal cord and brain stem (data not shown) or alter performance in the forced water swim test. This model of anxiety and depressive behavior is sensitive to changes in serotonin

abundance<sup>26</sup>. We suggest, therefore, that changes in serotonin production do not contribute to BH4-mediated increases in pain sensitivity. Because BH4 produces pain rapidly, it is likely that these immediate effects do not involve changes in transcription, activation of microglia<sup>42</sup> or induction of neuronal cell death<sup>9</sup>. Moreover, the efficacy of



**Figure 6** GTP cyclohydrolase haplotypes: association with pain and GCH1 regulation. **(a)** Locations of *GCH1* single nucleotide polymorphisms on coding DNA strand (numbered with 1 the A of start codon); those significantly associated with low pain scores are coded in red ( $*P < 0.05$ ). Letters in each haplotype are alleles for the 15 *GCH1* SNPs. Pain scores for each haplotype are the mean z-score for leg pain calculated from four questions assessing frequency of pain—at rest, after walking and their improvements after surgery—adjusted for covariates. Lower scores correspond to less pain. The highlighted haplotype (white) was associated with lower leg pain scores than the seven other haplotypes;  $P = 0.009$ . **(b)** Effect of number of copies of the pain-protective haplotype on frequency of leg pain at rest. O/O, X/O and X/X: individuals with 0, 1 and 2 copies of the haplotype, respectively. Numbers on y axis correspond to pain frequency: always (6), almost always (5), usually (4), ~half the time (3), a few times (2), rarely (1), not at all (0). Pre-op, before surgery. **(c)** Effect of number of copies of pain-protective haplotype (O/O  $n = 384$ ; X/O  $n = 153$  and X/X  $n = 10$ ) on experimental pain sensitivity in healthy volunteers (\*\* $P < 0.01$  compared with O/O group). **(d)** *GCH1* mRNA (QRT-PCR) in EBV-immortalized WBCs of O/O ( $n = 7$ ), X/O ( $n = 5$ ) and X/X ( $n = 4$ ) lumbar root pain study subjects stimulated with forskolin (10  $\mu\text{M}$ , 12 h), relative to unstimulated levels in O/O individuals (100%). White bars, unstimulated; gray, after stimulation. **(e)** Western blots showing GCH1 protein expression in immortalized WBCs and percentage change after forskolin. White bars, unstimulated; gray, after stimulation. **(f)** Biopterin in supernatants of forskolin-stimulated immortalized WBCs **(f)** and forskolin-stimulated whole blood from healthy volunteers **(g)**; O/O  $n = 11$ ; X/X  $n = 10$ ) relative to baseline (means  $\pm$  s.e.m.). Linear regression analysis showed significant effects of number of copies of pain-protective haplotype for forskolin-induced changes in *GCH1* mRNA ( $P < 0.001$ ), GCH1 protein ( $P = 0.037$ ) and biopterin ( $P = 0.001$  and  $P = 0.002$ ).

DAHP in the formalin test, peripheral inflammation and multiple models of neuropathic pain points to a common BH4-dependent mechanism in diverse pain conditions.

To evaluate the potential role of BH4 in human pain, we analyzed whether polymorphisms in the rate-limiting BH4-synthesizing enzyme GCH1 are associated with specific pain phenotypes. If BH4 is absent or very substantially reduced in humans due to rare missense, nonsense, deletion or insertion mutations in the coding regions of GTP cyclohydrolase<sup>43</sup> or sepiapterin reductase genes, DOPA-responsive dystonia and other severe neurological problems occur<sup>19,20</sup>. These neurological diseases are caused by dopamine or serotonin neurotransmitter deficiencies that result from the lack of BH4 as a cofactor for the enzymes that synthesize these transmitters. The homozygotes for the pain-protective haplotype in our study did not have any neurological diseases. We therefore speculated that the pain-protective haplotype embodies a variation in a regulatory site that causes a more modest impairment in GTP cyclohydrolase production or function. In support of this, we found that constitutive expression of GTP cyclohydrolase and BH4 production was equivalent in cells of carriers and noncarriers of the pain-protective haplotype. However, forskolin-evoked upregulation was significantly smaller in carriers of the pain-protective haplotype. Although the precise locations mediating the regulation of *GCH1* transcription have still to be determined, they likely involve elements in the region 5' to exon 1 and within the large 20 kb intron 1, because the SNPs found exclusively in the pain-protective haplotype are located in the putative promoter region of *GCH1* (C.-9610G>A) and in intron 1 (C.343+8900A>T), respectively. These SNPs might modify the efficiency of transcriptional modulation by signals mediated by cyclic AMP-dependent transcription factors. Although hundreds of transcripts are regulated in DRGs by nerve injury or sustained nociceptor stimulation, and although many chemical agents and biological molecules affect pain behavior in experimental settings, only a few genes have been identified so far that modulate pain sensitivity in humans<sup>11,44,45</sup>. The current finding for *GCH1* is one of the first to be replicated across three independent human study populations.

Our results demonstrate that alterations in the concentration of the essential enzyme cofactor BH4 modify the sensitivity of the pain system, and that SNPs in the gene for the rate-limiting BH4-producing enzyme GTP cyclohydrolase alter both responses in healthy humans to noxious stimuli and the susceptibility of patients to the development of persistent neuropathic pain. Because the pain-protective haplotype in *GCH1* is associated with a reduction in the risk of developing persistent pain without signs of dystonia, a treatment strategy that could reduce excess *de novo* synthesis in the DRG of BH4, but not constitutive concentrations of BH4, by targeting only induction of GTP cyclohydrolase or by leaving the recycling pathway intact, might prevent the establishment or maintenance of chronic pain. In addition, the identification of a predictor of the intensity and chronicity of pain will be a useful tool to assess an individual's risk for developing chronic pain. The effect of the pain-protective haplotype on both experimental and persistent pain, and the involvement of BH4 in both inflammatory and neuropathic pain, may explain why sensitivity to acute experimental pain is a predictor of postsurgical and eventually chronic pain<sup>46,47</sup>.

## METHODS

**Nociceptive models.** For the SNI model two branches of the rat sciatic nerve, the common peroneal and the tibial, were ligated and sectioned distally. In the CCI model the sciatic nerve was constricted with three ligatures; in the SNL model the L5 spinal nerve was tightly ligated. For the formalin test, 50  $\mu$ l of

5% formaldehyde solution were injected into a hindpaw and flinches were counted per minute up to 60 min. Paw inflammation was induced with 50  $\mu$ l complete Freund's adjuvant (CFA) injected into a hindpaw. Nociceptive analysis was conducted blinded and animals were fully habituated to the room and test cages. Mechanical allodynia was assessed with graded-strength monofilament von Frey hairs (0.0174–20.9 g, log scaled), cold allodynia with the acetone test and heat hyperalgesia with the Hargreaves test. Drugs (Sigma) were injected i.p. or i.t. through a spinal catheter; osmotic pumps were used for infusion. Control animals received vehicle. L4-5 DRG and spinal cord tissue was processed for QRT-PCR, western blotting, *in situ* hybridization and immunofluorescence studies (**Supplementary Methods** online). Animal procedures were approved by the Committee on Research Animal Care of the Massachusetts General Hospital.

**Concentrations of DAHP, neopterin and biopterin.** Concentrations were determined by liquid chromatography coupled to tandem mass spectrometry on a tandem quadrupole mass spectrometer (PE Sciex API 4000; Applied Biosystems). Biopterin and neopterin analysis is described at *Nature Protocols* online (DOI: 10.1038/nprot.2006.298); DAHP analysis is described in **Supplementary Methods**.

**Electrophysiology and calcium imaging.** Miniature excitatory postsynaptic currents were recorded at  $-70$  mV by whole-cell patch clamp in adult rat transverse spinal cord slices<sup>48</sup>.  $[Ca^{2+}]_i$  was measured fluorometrically as the ratio of the absorbances at 340 and 380 nm ( $\Delta F_{340/380}$ ) in cultured adult DRG neurons loaded with the  $Ca^{2+}$ -sensitive fluorescent dye Fura-2. BH4 (0.3–10  $\mu$ M), DEA-NONOate (50  $\mu$ M) and L-NAME (10–100  $\mu$ M) were applied using a multibarrel fast drug-delivery system.

**Immortalization of leukocytes and forskolin stimulation.** Peripheral blood lymphocytes were immortalized with EBV transfection. WBCs were stimulated with PHA in RPMI medium; EBV was then added and cells were incubated at 37 °C, 4.5% CO<sub>2</sub>, 90% relative humidity. Immortalized cells were stimulated with 10  $\mu$ M forskolin for 12 h.

**Data analysis.** Data are means  $\pm$  s.e.m. The number of animals per group was 9–12. Areas under the effect-versus-time curves were calculated using the linear trapezoidal rule and compared with Student's *t*-test or univariate analysis of variance (ANOVA) with subsequent *t*-tests employing a Bonferroni  $\alpha$ -correction for multiple comparisons. All other data were analyzed with univariate ANOVA or ANOVA for repeated measurements. *P* at 0.05 was considered significant for all tests.

**Human genetic studies.** We genotyped 15 single nucleotide polymorphisms (SNPs) spaced evenly through *GCH1* using the 5' exonuclease method (primer sets and probes in **Supplementary Table 3** online). *GCH1* haplotypes were identified *in silico* using the SAS/genetics software package (SAS Institute, Inc.), which implements a modified expectation-maximization algorithm to reconstruct haplotypes from population genotype data. Linkage disequilibrium between SNPs was used to describe the nonindependence of alleles (**Supplementary Fig. 4**).

**Chronic lumbar root pain.** We collected DNA from 168 Caucasian adults who participated in a prospective observational study of surgical discectomy for persistent lumbar root pain<sup>29</sup>. The primary endpoint was persistent leg pain over the first postoperative year, using four 'leg pain' variables (details in **Supplementary Methods**) normalized to z-scores with mean 0 and standard deviation 1. The primary pain outcome variable for each individual was the mean of these four z-scores. There were 147 subjects who completed the 1-year questionnaire. Genotype-phenotype associations for each SNP were sought by regression analysis. The covariates were a number of demographic, psychological and environmental factors, including sex, age, worker's compensation status, delay in surgery after enrollment and Short-Form 36 general health scale. Stepwise regression<sup>31</sup> was applied to assess the association between pain scores and haplotypes with frequencies >1%, obtained from the Ensemble database v.38, April 2006. These haplotypes accounted for 94% of chromosomes studied. If a haplotype was identified to be significantly (*P* < 0.05) associated with pain scores, phenotype-diplotype association analysis was

performed by regression analysis. The collection of DNA and genetic analyses were carried out with the approval of the National Institute of Dental and Craniofacial Research institutional review board and informed consent was obtained from all subjects.

**Experimental pain sensitivity in healthy subjects.** In two separate cohorts of healthy volunteers we analyzed the association of heat, ischemic and mechanical pain with *GCHI* haplotypes. One cohort was examined at the University of North Carolina at Chapel Hill and the second cohort was examined at the University of Florida. Experimental procedures used to assess pain perception are described in refs. 11,49. In order to combine the data across the two cohorts, each subject's value for a given pain measure was standardized to z-scores. Differences between the diplotype groups were determined using one way ANOVA followed by Bonferroni *post hoc* testing (*P* at 0.05). Results of the individual cohorts are in **Supplementary Table 4**. The studies were carried out with the approval of the institutional review boards of the University of North Carolina and University of Florida. Informed consent was obtained from all subjects.

Note: Supplementary information is available on the Nature Medicine website.

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#### AUTHOR CONTRIBUTIONS

I.T. and M.C. designed and organized experiments; performed animal studies, expression and function analyses and human screens; analyzed data; generated figures; and wrote the manuscript. R.S.G. contributed to the initial study concept and performed expression profiling and *in situ* hybridization. A.A. performed *in situ* hybridization and enzyme and antibody production studies. I.B. conducted human genotyping. H.S. designed and performed LC-MS/MS analyses. C.E. performed electrophysiology. J.N. conducted human lymphocyte studies. J.S. conducted animal studies and wrote the manuscript. C.M. conducted animal studies. T.W. analyzed spine pain data. A.A. studied animal behavior. L.D. analyzed human experimental pain data. A.M.B. performed calcium imaging studies. D.G. devised genotyping approaches and human lymphocyte studies. J.A. conducted haplotype function analysis. S.S. analyzed human experimental pain data. S.J.A. collected and adapted clinical spine data. W.A.C. and A.P. conducted the forced swim test. J.L. analyzed human genetic data and designed haplotype function analyses. R.B.F. and W.M. phenotyped experimental pain cohort and interpreted genetic data. G.G. initiated, organized and supervised analytical studies. M.B.M. initiated, organized and analyzed human studies and wrote the manuscript. C.J.W. initiated and supervised the study, designed experiments, analyzed data and wrote the manuscript.

#### COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Medicine* website for details).

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