Synaptic Plasticity in Spinal Lamina I Projection Neurons That Mediate Hyperalgesia

Hiroshi Ikeda,[1,2]* Bernhard Heinke,[1,2] Ruth Ruscheweyh,[1,2] Jürgen Sandkühler[1,2]†

Inflammation, trauma, or nerve injury may cause enduring hyperalgesia, an enhanced sensitivity to painful stimuli. Neurons in lamina I of the spinal dorsal horn that express the neurokinin 1 receptor for substance P mediate this abnormal pain sensitivity by an unknown cellular mechanism. We report that in these, but not in other nociceptive lamina I cells, neurokinin 1 receptor–activated signal transduction pathways and activation of low-threshold (T-type) voltage-gated calcium channels synergistically facilitate activity- and calcium-dependent long-term potentiation at synapses from nociceptive nerve fibers. Thereby, memory traces of painful events are retained.

Lamina I neurons that mediate hyperalgesia express the neurokinin 1 (NK1) receptor for substance P (*1*, *2*), which is released from primary afferent Aδ- and C-fibers upon intense noxious stimulation (*3*, *4*). Selective destruction of these neurons by saporin conjugated to substance P attenuates the development of hyperalgesia following inflammation of a hind paw or tight ligation of L5 and L6 spinal nerves, a model of neuropathic pain, in behaving rats (*1*, *2*). In anesthetized rats, sensitization of spinal nociceptive neurons by chemical excitation of C-fibers with capsaicin (*5*) is diminished. Many neurons in lamina I that express the NK1 receptor have an ascending projection to the brain (*6*), consistent with the involvement of a supraspinal loop in central sensitization (*7*). The cellular mechanisms by which these lamina I neurons mediate hyperalgesia and central sensitization are unknown.

Recordings were made from 516 lamina I neurons (*8*). Because only a subset of lamina I neurons express the NK1 receptor (*9*), we took advantage of the fact that 80% of all lamina I neurons that send a projection to the parabrachial area (*6*) express the NK1 receptor. We performed whole-cell patch-clamp recordings from 355 lamina I projection neurons (PNs) that were retrogradely labeled with DiI (Fig. 1, A and B). For comparison we also recorded from 161 unidentified neurons (UNs) in lamina I. Sixty-nine of 71 PNs and 37 of 48 UNs tested received mono- and/or polysynaptic input from fine primary afferent nerve fibers, most of which are nociceptive. This result is consistent with the finding that all spinoparabrachial neurons (*10*) and most lamina I neurons are nociceptive specific. To determine whether PNs express functional NK1 receptors, we added substance P to the bath solution in the presence of tetrodotoxin. This addition induced a transient inward current in 77% of 27 PNs tested (Fig. 1D). Responses were prevented by addition of the specific substance P receptor antagonist L-703,606 ($n = 9$) to the bath solution. In contrast, seven of nine UNs failed to respond to substance P (Fig. 1E). Lamina I projection neurons retrogradely labeled with DiI ($n = 85$) displayed significantly different active and passive membrane properties as compared with UNs ($n = 70$). These differences included larger membrane capacitance (69 ± 3 pF versus 37 ± 3 pF), more negative resting membrane potentials (mean ± SE, -63 ± 1 mV versus -58 ± 1 mV; $P < 0.01$, t test), and more negative thresholds for action potential (AP) firing (-43 ± 1 mV versus -37 ± 3 mV). To exclude the possibility that these differences were due to the incorporation of DiI, we also tested Fluoro Gold as a retrograde marker, which produced similar results (table S1).

We tested whether activity-dependent synaptic long-term potentiation (LTP), which is a potential cellular mechanism of afferent-induced hyperalgesia (*11*–*14*), can preferentially be induced in PNs. We measured mono- and polysynaptic C-fiber–evoked excitatory postsynaptic currents (EPSCs) from

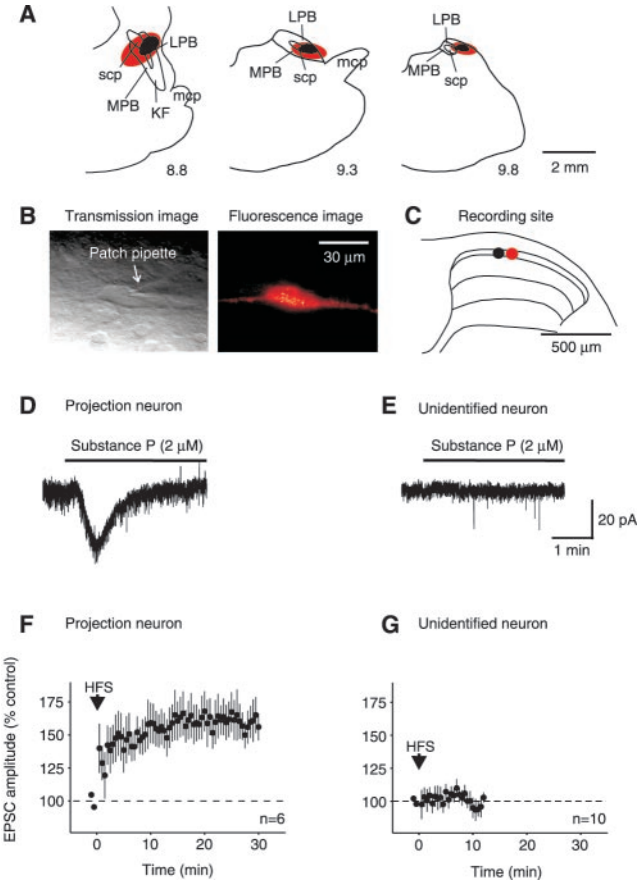

Fig. 1. Projection neurons in lamina I express the NK1 receptor. (**A**) The distribution of 200 nl DiI (2.5%) injected into the parabrachial area in one representative animal. KF, Kölliker Fuse subnucleus; LPB, lateral parabrachial area; MPB, medial parabrachial area; mcp, middle cerebellar peduncle; scp, superior cerebellar peduncle. Black indicates the area damaged by the injection; orange indicates the spread of the tracer into adjacent regions. The numbers under each section indicate the distance (in millimeters) posterior to bregma. (**B**) Lamina I projection neuron retrogradely labeled with DiI in transmission and in fluorescence mode. The location of this neuron is shown in (**C**) by an orange dot in the slice outline. The black dot indicates the UN from (E). PNs (**D**) but not UNs (**E**) respond with an inward current to bath application of substance P (2 μM) in the presence of tetrodotoxin (0.5 μM). (D) is from the same neuron as (B). EPSCs were measured in lamina I neurons in response to electrical stimulation of dorsal roots at C-fiber strength. Mean times courses EPSC amplitudes ± SE in PNs (**F**) and UNs (**G**) before and after conditioning HFS of the dorsal root at 100 Hz for 1 s three times at 10-s intervals.

[1]Institute of Physiology and Pathophysiology, Heidelberg University, D-69120 Heidelberg, Germany. [2]Brain Research Institute, Vienna University Medical School, A-1090 Vienna, Austria.

*Present address: Department of Human and Artificial Intelligence Systems, Fukui University, Fukui, Japan.
†To whom correspondence should be addressed. E-mail: juergen.sandkuehler@univie.ac.at

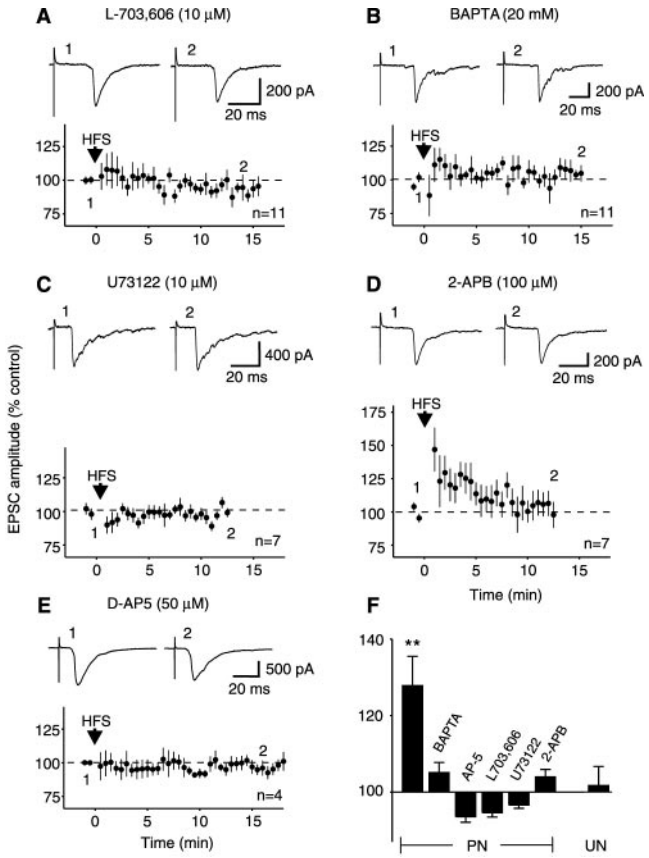

Fig. 2. Induction of LTP of synaptic strength between afferent C-fibers and PNs by HFS is blocked by the NK1 receptor antagonist L-703,606 (10 μM) (**A**), by the Ca^{2+} chelator BAPTA (20 mM, included in the pipette solution) (**B**), by the PLC inhibitor U73122 (10 μM) (**C**), by blocking IP_3 receptors with 2-APB (100 μM) (**D**), or by blocking NMDA receptor channels with D-AP5 (50 μM) (**E**), added to the bath solution. (**F**) Mean changes (+SE) in synaptic strength induced by HFS in PNs and UNs under control conditions and during application of drugs (PNs, control: 128 ± 8% of control; n = 23, $P < 0.01$; L-703,606: 95 ± 1% of control, n = 11; BAPTA: 105 ± 3% of control, n = 11; U73122: 97 ± 1% of control, n = 7; 2-APB: 104 ± 2% of control, n = 7; D-AP5: 94 ± 1% of control, n = 4; UNs: 102 ± 5% of control, n = 10). Traces show original EPSC recordings evoked by stimulation of a dorsal root; spontaneous EPSCs are occasionally superimposed. ** $P < 0.01$.

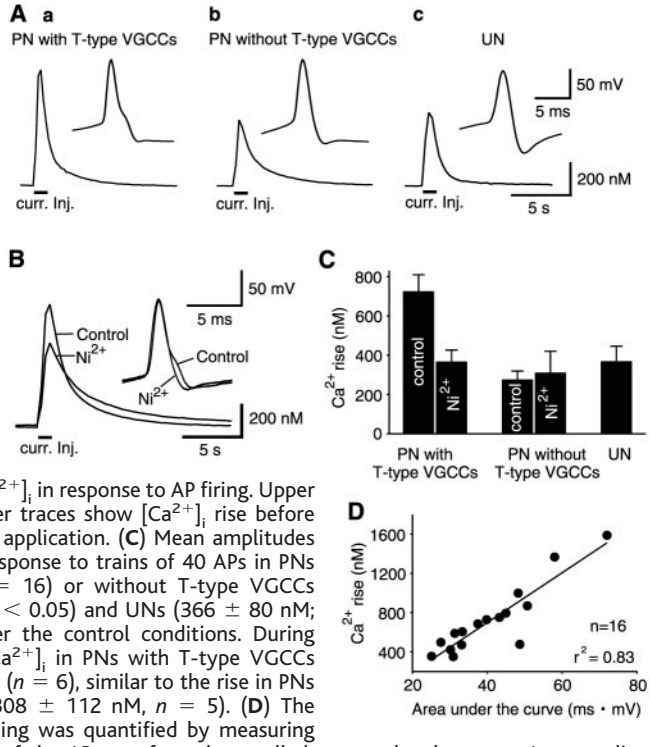

Fig. 3. AP broadening in PNs is correlated with the magnitude of activity-dependent rise in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). (**A**) Upper traces show APs; lower traces show rise in $[Ca^{2+}]_i$ in response to a train of 40 action potentials in 1 s for a PN with (a) and without (b) T-type VGCCs and a UN (c). (**B**) Blockade of T-type VGCCs by addition of Ni^{2+} (100 μM) to the bath solution abolished AP broadening and reduced the rise in $[Ca^{2+}]_i$ in response to AP firing. Upper traces show APs and lower traces show $[Ca^{2+}]_i$ rise before (control) and during Ni^{2+} application. (**C**) Mean amplitudes (+SE) of $[Ca^{2+}]_i$ rise in response to trains of 40 APs in PNs with (722 ± 88 nM, n = 16) or without T-type VGCCs (273 ± 47 nM; n = 18, $P < 0.05$) and UNs (366 ± 80 nM; n = 12, $P < 0.05$) under the control conditions. During superfusion with Ni^{2+}, $[Ca^{2+}]_i$ in PNs with T-type VGCCs increased to 365 ± 62 nM (n = 6), similar to the rise in PNs without T-type VGCCs (308 ± 112 nM, n = 5). (**D**) The magnitude of AP broadening was quantified by measuring the area under the hump of the AP waveform, here called area under the curve. A strong linear correlation exists between the size of the area under the curve in PNs and the amplitude of the $[Ca^{2+}]_i$ rise in response to AP firing.

PNs and UNs before and after applying conditioning high-frequency stimulation (HFS) to the spinal dorsal root. In 15 of 23 (65%) PNs tested, but in none of 10 UNs (Fig. 1, F and G), HFS induced LTP of C-fiber–evoked EPSCs. When results from all 23 recorded PNs were pooled, mean EPSC amplitudes significantly increased to 128 ± 8% ($P < 0.01$) and did not decrease within 30 min after HFS (Fig. 1F). We tested whether the supraspinal projection or the expression of NK1 receptors is a better marker for neurons that express synaptic plasticity. HFS induced synaptic LTP in all PNs that responded to bath application of substance P (2 μM) (mean EPSC amplitude 138 ± 6% of control at 10 min after HFS; n = 5). In these neurons, substance P induced a desensitizing inward current (21 ± 7 pA, holding potential V_{hold} = –55 mV), indicating that these neurons expressed functional NK1 receptors. In contrast, HFS failed to change synaptic strength in all five PNs that did not respond to substance P (supporting online text). We asked whether the expression of NK1 receptors is only a marker or whether it has any functional role for synaptic plasticity. In the presence of the NK1 receptor antagonist L-703,606, HFS of dorsal roots failed to induce synaptic LTP in all PNs tested (Fig. 2A). Activation of NK1 receptors may lead to a rise in the concentration of free cytosolic Ca^{2+} by Ca^{2+} release from inositol 1,4,5-trisphosphate (IP_3)–sensitive intracellular Ca^{2+} stores through the phospholipase C (PLC) pathway. Blockade of Ca^{2+} rise by BAPTA added to the pipette solution (Fig. 2B), blockade of PLC (Fig. 2C), or blockade of IP_3 receptors (Fig. 2D) all prevented LTP induction. Substance P may also enhance Ca^{2+} influx through NMDA (*N*-methyl D-aspartate) receptor channels in some cells. Bath application of substance P significantly and supraadditively increased NMDA receptor–mediated inward currents in PNs but not in UNs (fig. S1A) through the PLC pathway (fig. S1B). Pharmacological blockade of NMDA receptors prevented LTP induction by HFS (Fig. 2E). Thus, the presence and activation of NK1 receptors on spinal lamina I projection neurons are essential for the induction of activity-dependent synaptic LTP that requires a substance P–induced rise in Ca^{2+}, likely by Ca^{2+} release from intracellular stores, and a substance P–facilitated Ca^{2+} influx through NMDA receptor channels.

Upon excitation, 62 of 85 (73%) PNs tested, but virtually none (3%) of 70 UNs, discharged APs with a hump in the falling phase leading to AP broadening (Fig. 3Aa). To determine the ionic basis for AP broadening, we added Cd^{2+} to the bath solution, which nonselectively blocks voltage-gated calcium channels (VGCCs). Cd^{2+} abolished or strongly reduced AP broadening (n = 5,

$P < 0.05$). To identify the type of VGCC involved, we added the L-type VGCC blockers nicardipine or verapamil to the bath solution, which failed to affect AP broadening ($n = 5$ each, $P > 0.05$). In contrast, Ni^{2+}, which blocks T/R-type VGCCs when applied at micromolar concentrations, abolished or strongly reduced AP broadening ($n = 8$, $P < 0.01$), suggesting that opening of T/R-type VGCCs is involved (Fig. 3, B and C). To examine whether low voltage–activated T-type and/or high voltage–activated R-type Ni^{2+}-sensitive calcium currents (*15*) are differentially expressed in neurons with or without AP broadening, we determined the activation curves (*8*). In all 11 neurons with AP broadening, T-type VGCCs with low voltage-activation thresholds (–65 mV from a V_{hold} of –90 mV) were identified. None of seven neurons without AP broadening displayed a T-type calcium current. The expression of T-type VGCCs in PNs with AP broadening, but not in other lamina I neurons, suggests that these neurons have a stronger activity-dependent influx of Ca^{2+}. We thus measured cytosolic Ca^{2+} transients in the somata of lamina I neurons, using Fura-2 in the pipette solution during controlled AP discharges (*8*). Short depolarizing current pulses were adjusted to elicit a single AP per pulse and were applied at 40 Hz for 1 s. Neurons with T-type VGCCs exhibited significantly higher Ca^{2+} transients during AP discharges than other lamina I neurons (Fig. 3, A and C). In the presence of Ni^{2+}, however, the Ca^{2+} rise in neurons with T-type VGCCs was not different from those without (Fig. 3C). The size of AP broadening quantified as the area under the hump of the AP waveform was positively and linearly correlated with Ca^{2+} rise (Fig. 3D). Thus, opening of T-type VGCCs during AP firing enhanced Ca^{2+} influx in these neurons.

An indirect indication that T-type VGCCs are involved in LTP induction is the observation that in only one of five PNs without T-type VGCCs, but in all five PNs with T-type VGCCs, LTP could be induced by HFS (Fig. 4, A and B). The expression of T-type VGCCs in lamina I neurons was associated with a steeper Ca^{2+} rise during conditioning HFS (Fig. 4, C and D). The strength in Ca^{2+} rise was a good predictor of the magnitude of LTP (Fig. 4E). To directly examine whether the induction of this form of synaptic LTP requires Ca^{2+} influx into postsynaptic cells through T-type VGCCs, we included Ni^{2+} in the bath solution, which abolished LTP induction (Fig. 4G).

The present study identified synaptic mechanisms that lead to activity-dependent sensitization of spinal dorsal horn lamina I neurons that mediate abnormal sensitivity to pain. Induction of LTP by conditioning stimulation of C-fibers required coactivation of NK1 receptors for substance P, Ni^{2+}-sensitive T-type calcium currents, and NMDA receptors, all contributing to a steep rise in postsynaptic Ca^{2+} levels that may be required for LTP induction (*16*, *17*). All three cloned members of the T-type VGCC family (α1G, α1H, and α1I) are present in the spinal dorsal horn, with the α1G and α1H subtypes being most prominent in lamina I (*18*). Blocking spinal T-type VGCCs with ethosuximide depressed all types of sensory responses of spinal dorsal horn neurons in vivo (*19*). A role of T-type VGCCs in the sensitization of spinal nociceptive neurons had not been shown previously. Hyperalgesia in behaving animals involves calcium-dependent signal transduction pathways: Animals with neuropathic pain have enhanced cytosolic Ca^{2+} concentrations in spinal neurons (*20*). Neuropathic but not acute pain requires activation of calcium-activated protein kinase C or calcium-calmodulin–dependent protein kinase II. This activation may lead to phosphorylation of GluR1 subunit of the AMPA receptor, which enhances glutamatergic synaptic transmission (*11*, *21*, *22*). LTP can also be induced in intact rats by electrical stimulation of dorsal roots (*23*) or by natural noxious stimulation including inflammation, trauma, and nerve injury (*24*). LTP induction in vivo also requires coactivation of NK1 and NMDA receptors (*23*). An LTP-like, NMDA receptor–dependent enhancement of pain sensitivity has recently been induced in human volunteers by high-frequency conditioning transcutaneous electrical nerve stimuli (*25*). Thus, LTP at synapses between nociceptive afferents and a well-defined group of neurons in the spinal dorsal horn underlies some forms of abnormal sensitivity to pain.

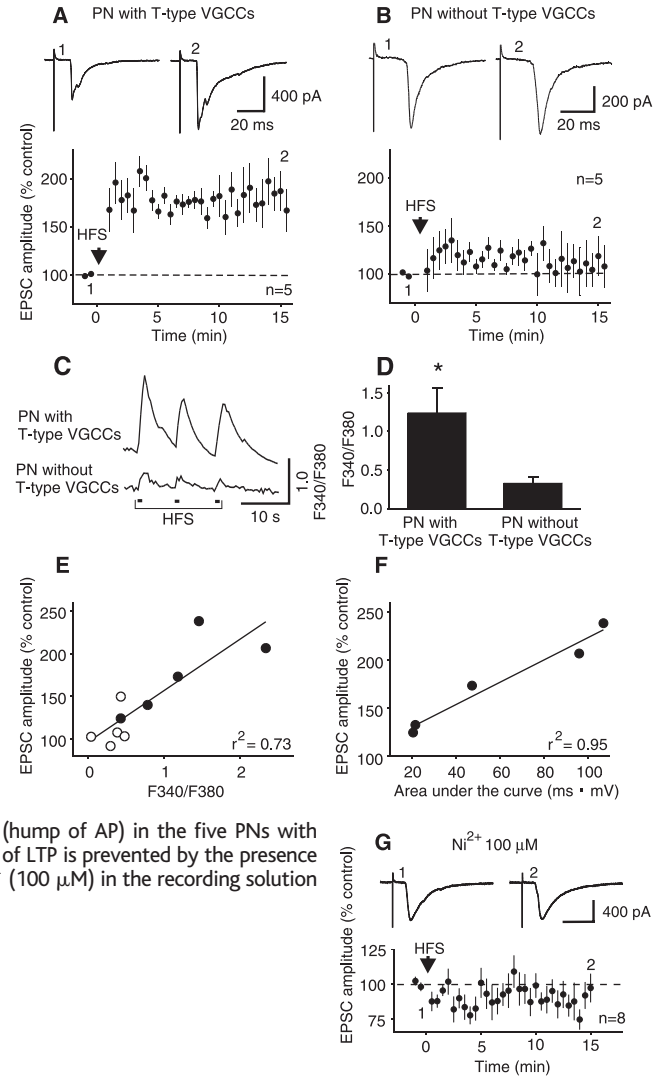

Fig. 4. LTP induction of C-fiber–evoked EPSCs requires activation of T-type VGCCs. LTP is reliably evoked by HFS in PNs with (**A**) but not without (**B**) T-type VGCCs. (**C**) Examples of Ca^{2+} signals of PNs with and without T-type VGCCs during HFS (F340/F380: ratio of the intensities of fluorescence measured at 340 nm and 380 nm). (**D**) The $[Ca^{2+}]_i$ rise during HFS was quantified by addition of the F340/F380 values of the three Ca^{2+} peaks evoked by HFS. The $[Ca^{2+}]_i$ rise during HFS in PNs with T-type VGCCs was significantly larger (1.2 ± 0.3, $n = 5$) than that in PNs without T-type VGCCs (0.3 ± 0.1; $n = 5$, $P < 0.05$). (**E**) A linear correlation exists between the change of EPSC amplitude and the $[Ca^{2+}]_i$ rise during HFS [(●), PNs with, (○), PNs without T-type VGCCs]. (**F**) A linear correlation exists between the change of EPSC amplitude following HFS and the size of the area under the curve (hump of AP) in the five PNs with T-type VGCCs. (**G**) Induction of LTP is prevented by the presence of T-type VGCC blocker Ni^{2+} (100 μM) in the recording solution ($n = 8$). * $P < 0.05$.

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Supporting Online Material
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COMT *val*^{158}*met* Genotype Affects μ-Opioid Neurotransmitter Responses to a Pain Stressor

Jon-Kar Zubieta,[1,2]* Mary M. Heitzeg,[1] Yolanda R. Smith,[3] Joshua A. Bueller,[1] Ke Xu,[5] Yanjun Xu,[1] Robert A. Koeppe,[2] Christian S. Stohler,[4] David Goldman[5]

Responses to pain and other stressors are regulated by interactions between multiple brain areas and neurochemical systems. We examined the influence of a common functional genetic polymorphism affecting the metabolism of catecholamines on the modulation of responses to sustained pain in humans. Individuals homozygous for the *met*^{158} allele of the catechol-*O*-methyltransferase (COMT) polymorphism (*val*^{158}*met*) showed diminished regional μ-opioid system responses to pain compared with heterozygotes. These effects were accompanied by higher sensory and affective ratings of pain and a more negative internal affective state. Opposite effects were observed in *val*^{158} homozygotes. The COMT *val*^{158}*met* polymorphism thus influences the human experience of pain and may underlie interindividual differences in the adaptation and responses to pain and other stressful stimuli.

Strong interest exists for the discovery of genes that cause individual differences in responses to physical and environmental challenges. In the case of pain, both sensitivity and inhibition are traits that vary considerably among individuals, with some of the variability being attributed to genetic factors (*1*, *2*). However, the influence of genes on regulatory processes in the human brain is particularly difficult to resolve. A functional genetic variant may affect not only the protein coded by the gene in question but may also have downstream effects contributing to the overall system response. Furthermore, differences in human resiliency and stress responses determine individual vulnerabilities to many psychiatric and other complex diseases (*3*, *4*).

[1]Department of Psychiatry and Mental Health Research Institute, [2]Department of Radiology, [3]Department of Obstetrics and Gynecology, Medical School, and [4]Department of Biologic and Materials Sciences, School of Dentistry, The University of Michigan, Ann Arbor, MI 48109–0720, USA. [5]Laboratory of Neurogenetics, National Institute of Alcohol and Alcoholism, Rockville, MD 20852, USA.

*To whom correspondence should be addressed. E-mail: zubieta@umich.edu.

Here, we focus on an abundant functional polymorphism of the cathechol-*O*-methyltransferase (COMT) gene that codes the substitution of valine (*val*) by methionine (*met*) at codon 158 (*val*^{158}*met*). This substitution is associated with a difference in thermostability leading to a three- to fourfold reduction in the activity of the COMT enzyme (*5*). The alleles are codominant so that individuals with the *val/val* genotype have the highest activity of COMT, those with the *met/met* genotype have the lowest activity of COMT, and heterozygous individuals are intermediate. The *val*^{158}*met* genotypes have been linked to a number of behavioral diseases of complex etiology (*6–10*).

COMT is one of the enzymes that metabolizes catecholamines, thereby acting as a key modulator of dopaminergic and adrenergic/noradrenergic neurotransmission (*11*, *12*). Different levels of COMT activity conferred by *val*^{158}*met* genotypes may then have important influences on functions regulated by these neurotransmitters, including μ-opioid system responses. In animal models, the chronic activation of dopaminergic neurotransmission and D2 receptors, a situation parallel to that encountered in *met/met* homozygotes, reduces the neuronal content of enkephalin peptides and induces compensatory increases in regional μ-opioid receptor concentrations in various brain regions. Reductions in D2 receptor–mediated neurotransmission, similar to that achieved by the higher levels of COMT activity in *val/val* homozygotes, results in opposite effects on the μ-opioid system (*13–16*). Therefore, we hypothesized that chronic overactivity of the dopaminergic system induced by the low-function *met/met* COMT enzyme would be associated with a lesser capacity to activate μ-opioid neurotransmission under provocation conditions by virtue of a lower neuronal content of enkephalin. Compensatory increases in μ-opioid receptor binding should also be observed under these circumstances. We hypothesized intermediate effects in heterozygous individuals, while the presence of the higher metabolic capacity of the *val/val* COMT genotype would be associated with higher enkephalin content, a superior capacity to activate μ-opioid neurotransmission and possibly compensatory reductions in receptor binding levels. The μ-opioid system is activated in response to stressors, pain, and other salient environmental stimuli, typically reducing pain and stress responses (*1*, *17–22*). At a psychophysical level, we expected the variations in the magnitude of μ-opioid system activation induced by the COMT genotypes to result in varying capacities to suppress responses to pain and other stressors.

To elucidate the contribution of *val*^{158}*met* COMT genotypes on the functional responses of the μ-opioid system and their psychophysical correlates, we exposed human subjects to a sustained pain challenge. As even moderate levels of pain become temporally more sustained, it becomes a significant physical and emotional stressor (*23*) and activates suppressive μ-opioid neurotransmitter responses (*1*, *24*). This activation is then associated with reductions in sensory and affective qualities of pain and in the negative affective state of the volunteers (*1*, *24*).

We studied 29 healthy volunteers (15