Capsaicin upregulates HDAC2 via TRPV1 and impairs neuronal maturation in mice

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Transient receptor potential vanilloid 1 (TRPV1) affects mood and neuroplasticity in the brain, where its role is poorly understood. In the present study we investigated whether capsaicin (8-methyl-N-vanillyl-trans-6-nonenamide), an agonist of TRPV1, induced chromatin remodeling and thereby altered gene expression related to synaptic plasticity. We found that capsaicin treatment resulted in upregulation of histone deacetylase 2 (HDAC2) in the mouse hippocampus and HDAC2 was enriched at *Psd95*, *synaptophysin*, *GLUR1*, *GLUR2* promoters. Viral-mediated hippocampal knockdown of HDAC2 induced expression of Synapsin I and prevented the detrimental effects of capsaicin on Synapsin I expression in mice, supporting the role of HDAC2 in regulation of capsaicin-induced Synapsin I expression. Taken together, our findings implicate HDAC2 in capsaicin-induced transcriptional regulation of synaptic molecules and support the view that HDAC2 is a molecular link between TRPV1 activity and synaptic plasticity.

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INTRODUCTION
Capsaicin (8-methyl-N-vanillyl-trans-6-nonenamide), a component of red chili peppers, is an agonist of transient receptor potential vanilloid type1 (TRPV1) whose activation increases pain by exciting primary sensory neurons in the peripheral nervous system.1 TRPV1 activation by agonists induces L-type calcium channel-dependent apoptosis of primary rat cortical neurons2 and impairs proliferation of neural progenitor cells in the hippocampus of young mice.3 These findings suggest that capsaicin is harmful to neural development in the brain. However, the effects of capsaicin on neuronal maturation and synaptic plasticity have not been adequately explored.

Preclinical studies show that TRPV1 mediates anxiety- and depression-related behaviors. TRPV1 activation by systemic injection of agonists such as capsaicin and resiniferatoxin elicits anxiogenic responses and depression-related behaviors, whereas its antagonists induce anxiolytic- and antidepressant-like effects in rodents.4–7 Besides, direct injection of capsaicin or capsazepine in the hippocampus induces anxiety- or anxiolytic-like effects.8,9 These effects are accompanied by impaired hippocampal neurogenesis through epigenetic regulation.10,11 A number of previous studies have demonstrated that chromatin-remodeling factors including histone deacetylases (HDACs) play a key role in adult neurogenesis and synaptic plasticity and hence in cognition and mood.12,13 HDACs repress gene transcription by removing acetyl groups from histone tails.12 We recently demonstrated that the antidepressant-like effects induced by TRPV1 deficiency are associated with HDAC2 downregulation.7 However, TRPV1 activation produces depression-like effects with an increase in HDAC2 expression. However, there is no evidence that TRPV1 activation by agonists induces HDAC2 upregulation and thereby alters synaptic molecules or neuronal maturation in the hippocampus.

We now report that systemic capsaicin administration for 2 weeks in adult mice led to a reduction in number of mature neurons as well as of neural proliferation in the dentate gyrus (DG) of the hippocampus. It also elevated the amount and activity of HDAC2 in the hippocampus, which in turn decreased synaptic plasticity-related molecules, as HDAC2 repressed the activity of their promoters. To investigate the effect of HDAC2 on synaptic density, we infected primary neurons...
hippocampal neurons with lenti-shHDAC2 to reduce HDAC2 expression. We found that knockdown of HDAC2 reversed the decrease in synaptic density induced by TRPV1 activation. Taken together, these results suggest that TRPV1 activation impairs synaptic plasticity by upregulating HDAC2.

MATERIALS AND METHODS

Mice
All experiments were conducted with 8–10-week-old male C57BL/6J (Charles River Korea, Seoul, Korea) mice. Trpv1⁻/⁻ mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). They were backcrossed into the C57BL/6J (Charles River Korea) background, and mice of the N7 or higher generation were used. Heterozygous breeders were crossed to generate wild type, heterozygous and knockout litters, and genotypes were determined using PCR analysis. Mice were housed four per cage in a temperature- and humidity-controlled environment (lights on 0700–1900) with access to food and water ad libitum. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Hanyang University and were performed in accordance with relevant guidelines and regulations.

Culture of primary hippocampal neurons
Hippocampi from day 14 C57BL/6 mouse embryos were rapidly and aseptically removed from brains into ice-cold Ca²⁺/Mg²⁺-free Hanks’ balanced salt solution (HBSS), followed by removal of meninges and mincing into small pieces. The hippocampal tissue was then digested with 0.25% EDTA-trypsin (Gibco, Grand Island, NY, USA) and mincing into small pieces. The hippocampal tissue was then digested with 0.25% EDTA-trypsin (Gibco, Grand Island, NY, USA) and mincing into small pieces. The hippocampal tissue was then digested with 0.25% EDTA-trypsin (Gibco, Grand Island, NY, USA) and mincing into small pieces. The hippocampal tissue was then digested with 0.25% EDTA-trypsin (Gibco, Grand Island, NY, USA) and mincing into small pieces. The hippocampal tissue was then digested with 0.25% EDTA-trypsin (Gibco, Grand Island, NY, USA) and mincing into small pieces. The hippocampal tissue was then digested with 0.25% EDTA-trypsin (Gibco, Grand Island, NY, USA) and mincing into small pieces. The hippocampal tissue was then digested with 0.25% EDTA-trypsin (Gibco, Grand Island, NY, USA) and mincing into small pieces.

| Table 1 Primary antibodies used in WB, ICC, IHC and ChIP. |
|-----------------------------------------------|
| **Antigen** | **Manufacturer** | **Species, type** | **Catalog number** | **Dilution** |
| NeuN | Chemicon/CA, USA | Mouse monoclonal | MAB377 | 1:100 |
| BrdU | Abcam/Cambridge, UK | Rat monoclonal | ab6326 | 1:300 |
| HDAC1 | Abcam/Cambridge, UK | Rabbit polyclonal | ab53091 | 1:1000 |
| HDAC2 | Abcam/Cambridge, UK | Rabbit polyclonal | ab16032 | 1:1000 |
| HDAC4 | Abcam/Cambridge, UK | Rabbit polyclonal | ab12172 | 1:1000 |
| HDAC5 | Abcam/Cambridge, UK | Rabbit polyclonal | ab1439 | 1:1000 |
| β-actin | Santa Cruz/TX, USA | Mouse monoclonal | sc-47778 | 1:2000 |
| pSer10acK14 Histone H3 | Millipore/CA, USA | Rabbit polyclonal | #07-081 | 1:1000 |
| Acetyl-Histone H3 | Millipore/CA, USA | Rabbit polyclonal | #06-599 | 1:1000 |
| Histone H3 | Upstate/NY, USA | Mouse monoclonal | #05-499 | 1:1000 |
| GluR1 | Millipore/CA, USA | Rabbit polyclonal | #06-306 | 1:1000 |
| GluR2 | Millipore/CA, USA | Rabbit polyclonal | AB1768-I | 1:1000 |
| NR1 | Upstate/NY, USA | Rabbit monoclonal | #06-311 | 1:1000 |
| PSD95 | Abcam/Cambridge, UK | Rabbit polyclonal | ab18258 | 1:1000 |
| MAP2 | Sigma-Aldrich/MO, USA | Mouse monoclonal | M1406 | 1:500 |
| Synapsin1 | Cell Signaling/MA, USA | Rabbit monoclonal | #5297 | 1:300 |
| GFP | Santa Cruz/TX, USA | Rat monoclonal | sc-101536 | 1:200 |

Abbreviations: BrdU, bromodeoxyuridine; ChIP, chromatin immunoprecipitation; HDAC, histone deacetylase; ICC, immunocytochemistry; IHC, immunohistochemistry; NeuN, neuronal nuclei; WB, western blotting.
Table 2 Primer sequences for qPCR.

| Gene          | 5'-Forward-3'                        | 5'-Reverse-3'                        |
|---------------|--------------------------------------|--------------------------------------|
| Actb (Actin)  | GCCGCTGTGGCGTCTCTATAA                 | TGGAGGTACTAGCCACGGAGAG                |
| Synapsin2     | GGTCTTCTTCCCTCCACAC                   | CAGACAGACTACACAGAAAG                 |
| Synaptophysin | CTAGCTCTCAGGAAGAAATG                  | GGGAAGAGGAGAAGAGTGTG                 |
| Pdcd5         | CCCATCCCTCCTGGAATAT                   | TTTCGCAATTCCCTGCTT                  |
| GLUR1         | GAGGAGAGCACAGAGGAGAG                  | AGCAAGGAGGCCGGAGCT                   |
| GLUR2         | CCCTCGATTAACTGAAATG                   | CACAGCTTGCGGCTTAT                    |
| Fos           | GAAAGCCTGGGCTGATG                    | ACGAAAGAGCAGGGAACT                  |
| Creb          | CTACCCACCTCTCCGAGT                   | AGCAAGGAGCAGGGGCA                   |
| Hdlc2 (mRNA)  | AAATCAGCTCAGAAGGCA                    | GTCGTTGAGAGCAAGAG                   |
| Synapsin1     | CACCAGAGATGAGAGGAGC                   | ACCACACGTGTGCGCATCTA                 |

Abbreviation: qPCR, quantitative real-time PCR.

Wetlar, Germany) at ×400, plus video camera and LEICA IM50 software (Leica Microsystems) in six-to-eight coronal 25 μm sections spaced 400 μm apart per mouse. BrdU (+) cells in SGZ and granular cell layer sections were counted by an experimenter blind to the study code. Data are expressed as the average number of immunoreactive cells, and reported as means ± s.e.m. For statistical analysis, unpaired t-tests were used.

**Extraction of histones**
Histones were acid-extracted as described previously. Hippocampi were homogenized in lysis buffer (β#803, Cell Signaling, Danvers, MA, USA) and the pellets were resuspended in 0.4 N H2SO4 and incubated on ice for 20 min. Samples were washed with acidified acetone and centrifuged at 14 000 r.p.m. The histone-containing pellets were dissolved in 10 mM Tris (pH 8.0) and subjected to western blot analysis.

**Immunohistochemistry and western blot analysis**
Mice were perfused with 4% paraformaldehyde in PBS for 20 min and processed for histology. Brains were rapidly isolated and immediately frozen and stored at −70 °C. Serial sections (25 μm per section) were cut coronally through the entire anteroposterior extent of the hippocampus. To detect BrdU and neuronal nuclei (NeuN), sections were processed as described. For other types of immunofluorescence labeling, sections were incubated in 10% normal goat serum and 0.3% Triton X-100 for 1 h at room temperature. They were then incubated overnight at 4 °C in 0.1 M PBS, pH 7.4, containing 0.3% Triton X-100 (PBST), and washed in PBST. Primary antibodies used for immunohistochemistry are described in Table 1. To detect primary antibodies, cells were incubated in PBST containing Cy3-labeled (Jackson Immunoresearch, West Grove, PA, USA), Cy5-labeled (Jackson Immunoresearch) or fluorescein isothiocyanate (FITC)-labeled (Invitrogen, Carlsbad, CA, USA) secondary antibodies for 2 h at room temperature (25 °C) and photographed with a confocal microscope (Leica Microsystems). Z-stack images were acquired by optical sectioning, and LAS AF software (Leica Microsystems) was used to sharpen images, adjust brightness and contrast levels, and compose Z-projection (maximum intensity) and channel-merged figures. Cells were counterstained with 4′,6-diamidino-2-phenylindole in addition to the four immunological markers. For western blot analysis, samples were prepared as previously described. The primary antibodies used for western blot analysis are described in Table 1. Chromatin immunoprecipitation assays
Chromatin immunoprecipitation (ChIP) assays were performed as previously described. The antibodies used are presented in Table 1. Immunoprecipitated DNA samples were resuspended in distilled H2O and used for real-time PCR (CFX96 Touch Real-Time PCR Detection System, Bio-Rad Laboratories, Foster City, CA, USA). Primer sequences are given in Table 2, and were previously reported. Input and immunoprecipitated DNAs were PCR-amplified in triplicate in the presence of SYBR Green (Bio-Rad Laboratories). The fraction of genomic DNA co-precipitated with specific antibody was calculated as: Δ Ct = Ct (genomic input) − Ct (specific antibody), where Ct (genomic input) and Ct (specific antibody) are the mean threshold cycles of PCR performed in duplicate on DNA samples from the genomic input samples and the antibody-treated samples, respectively. 2ΔCt target values were calculated for each antibody. Enrichment was assessed by quantitative real-time PCR (qPCR) for the seven promoters and normalized to the values obtained with the Actin promoter.

**RT-PCR and qPCR**
RNA was extracted from hippocampi with Trizol reagent (Sigma). Reverse transcription was performed with Improm-II (Promega), 1 μg of total RNA and oligonucleotide-dT primer. qPCR was performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories). The primers used to amplify cDNAs are given in Table 2. Ct values for each sample were obtained using CFX Manager Software version 3.0 (Bio-Rad Laboratories). The expression of each gene was normalized to the amount of β-actin. Normalized expression values were averaged, and average fold changes were calculated.

**Construction of a shRNA (shRNA-expressing vector) and lentivirus production**
For gene silencing, short hairpin RNAs (shRNAs) were cloned into the pLB lentiviral vector (Addgene, Cambridge, MA, USA), as described. Their sequences were as follows: Luciferase shRNA (shLuc, control shRNA), 5′-CTCCGAATGTCCGTTTGTTT-3′; HDAC2 shRNA (shHDAC2) 5′-CCCCATGTTGGCCATATATTA-3′. Lentivirus was produced by co-transfection of shLuc, shHDAC2 with the compatible packaging plasmids, pMD2.G and psPAX2 into 293T cells (ATCC, Manassas, VA, USA) by the calcium–phosphate method, as described by the RNAi Consortium (http://www.broadinstitute.org).
Statistical analyses

Data distributions were checked for normality and equality of variance. All experiments were carried out at least three times, and data consistency was observed in repeated experiments. Wherever possible, the experimenter conducting the data analysis was blind to the treatment conditions of the animals. Differences between groups were analyzed by two-way analysis of variance followed by the Bonferroni adjustment for multiple comparisons between groups when assessing the effect of drugs on the virus. GraphPad Prism5.0 (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis. $P<0.05$ was considered statistically significant. Biochemical data are presented as means±s.e.m. of multiple independent experiments performed in triplicate.

RESULTS

Capsaicin induces HDAC2 in the mouse hippocampus

We investigated whether capsaicin administration changed the amounts of HDAC1, 2, 4 and 5 in the mouse hippocampus in which these HDACs are abundant.$^{20}$ To investigate the threshold dose of capsaicin for inducing epigenetic effects in the hippocampus, we examined the dose-dependent effects of capsaicin administration. We found that HDAC2 expression had a tendency to increase in a dose-dependent manner with a significant increase at the dose of 0.1 mg kg$^{-1}$ (Figure 1a and b). Therefore, we used a 0.1 mg kg$^{-1}$ dose of capsaicin for 2 weeks, which also induced impairment of adult hippocampal neurogenesis as shown in a previous study.$^{3}$ HDAC2 protein increased upon capsaicin treatment compared with vehicle-treated controls (Figure 1c and d), whereas HDAC4 protein increased only slightly (Figure 1c and d). To ask whether the increases in HDAC2 and 4 were dependent on capsaicin-induced TRPV1 activation, we administered capsaicin to Trpv1$^{-/-}$ mice. Levels of HDAC2 and 4 in the hippocampus of Trpv1$^{-/-}$ mice were unaffected by capsaicin administration (Figure 1e and f), indicating that the increased levels of HDAC2...
and 4 were the result of TRPV1 activation. We then tested the activities of HDAC2 and class II HDACs including HDAC4. HDAC2 activity increased, but the activity of class II HDACs was not altered (Figure 2a and b). Because HDAC2 activity was elevated, its target substrate, histone H3, was less acetylated (Figure 2c and d). However, the level of histone H3 Ser10 and acetylation at Lys14 (pSer10-acK14H3), known to be associated with hippocampal learning and memory processes,21 was not altered by capsaicin (Figure 2c and d). These results demonstrated that elevation of HDAC2 regulated histone modification in the hippocampus through TRPV1-dependent signaling.

Capsaicin downregulates levels of postsynaptic molecules through HDAC2 in the hippocampus

A previous report has suggested that HDAC2 represses synaptic plasticity-related genes by binding to their promoters.17 Even though HDAC2 lacks a DNA-binding domain, its transcriptional repressor complex including CoREST and mSin3A has a role in gene silencing as suggested previously.22 Having seen that capsaicin increased HDAC2 in the hippocampus, we explored its effect on HDAC2 binding to the promoters of synaptic molecules. We examined the association of HDAC2 with the promoter regions of synapsin2 (Syn2), synaptophysin (SYP), GLUR1, GLUR2 and Psd95, and included the QUOTE β-actin promoter as control. Capsaicin increased HDAC2 binding to the SYP, GLUR1 and GLUR2 promoters (Figure 3a). We also noted that HDAC2 had a strong tendency to bind to the Psd95 promoter in the capsaicin-treated hippocampus (Figure 3a). Consistent with the repression of HDAC2 by capsaicin in the mouse hippocampus, we observed reduced levels of PSD95, GluR1 and GluR2 (Figure 3b and c). We further found that TRPV1 activation affected the binding of HDAC2 to the promoters of Fos and Creb (Figure 3a), which play an important role in neuronal differentiation and neurogenesis.23 Taken together, our findings indicated that increasing HDAC2 by capsaicin treatment led to a reduction of synaptic molecules.

Capsaicin reduces synaptic density in primary hippocampal neurons

To assess the effect of an increase in HDAC2 triggered by capsaicin on synaptic plasticity, we infected primary hippocampal neurons with lentishHDAC2 (Figure 4a) and confirmed that HDAC2 expression was attenuated at the mRNA level (Figure 4b). In addition, capsaicin alone increased Hdac2 mRNA levels in neurons infected with the control virus (Figure 4b). This upregulation of Hdac2 transcripts was accompanied by reduced Synapsin I transcription (Figure 4c). Capsaicin reduced the number of synaptic puncta, and this reduction was prevented by knockdown of HDAC2 (Figure 4d and e). These results indicated that capsaicin disrupted synaptic function by increasing HDAC2.

Capsaicin impairs the maturation as well as the proliferation of neurons in the DG

To further demonstrate the effect of capsaicin-induced TRPV1 activation on neurogenesis including neuronal proliferation and differentiation, we administrated BrdU to mice for three consecutive days before administering capsaicin for 2 weeks (Figure 5a) and examined the number of BrdU-positive cells in the SGZ and DG (Figure 5b). Capsaicin reduced the proliferation in the SGZ (Figure 5c) and survival of newborn neurons in the DG of adult mice (~23%; Figure 5d). We then examined expression of nuclear antigen (NeuN), a marker of neuronal maturation. The number of NeuN-positive cells co-labeled with BrdU was reduced by capsaicin (~36%, Figure 5e; ~31%, Figure 5f). These results suggest that capsaicin-induced TRPV1 activation impaired neuron maturation in the DG of adult mice.

DISCUSSION

Here we have shown that TRPV1 activation induced by capsaicin impaired neuron maturation and synaptic plasticity in the hippocampus. We suggest that capsaicin-induced TRPV1 activation upregulates HDAC2 and decreases acetylation of histone H3, resulting in reduced expression of synaptic molecules. Furthermore, TRPV1 activation leads to loss of synapses, which is reversed by knocking down HDAC2 in primary hippocampal neurons.

Previous work has revealed that animals treated with TRPV1 agonists and antagonists exhibit depression- and antidepressant-like behaviors, respectively.4–6,24,25 However, the molecular mechanisms that underlie these emotional responses have not been elucidated. There are a number of...
reports of the functional roles of HDAC2 in the maturation of neural cells,17 especially in neurogenesis and synaptic plasticity. According to our recent work, TRPV1 loss of function induces downregulation of HDAC2 in the DG, leading to elevated expression of neurogenesis- and synaptic plasticity-related genes, which contributes to stress-resilient behavior. However, increase of HDAC2 expression in the DG induces depression-like behaviors, which were blocked by HDAC2 knockdown.7 In line with this, we confirmed above that TRPV1 activity stimulated HDAC2 upregulation and impaired neuronal maturation. We also found that TRPV1 activity reduced the survival of neural progenitor cells as well as neural proliferation in the DG. Taken together, these results suggest that TRPV1 regulates neurogenesis and synaptic plasticity through HDAC2-mediated chromatic remodeling.

TRPV1 is mainly expressed in the sensory neurons of the peripheral nervous system as well as the hypothalamus, amygdala and hippocampus in the central nervous system.29 Systemic injection of capsaicin may produce pain-related effects through TRPV1 expressed in sensory neurons. These sensory inputs from the periphery terminate in the spinal cord, where integration and hypersensitivity can be established. Spinal outputs connect with the limbic system including the hippocampus.30 However, the capsaicin dosage (0.1 mg kg−1, intraperitoneally) used in our experiment is low relative to that used in pain induction or behavioral response change.31 Therefore, the indirect action of capsaicin in our study may not be significant.

In humans, TRPV1 may not be exposed to the dose of capsaicin that was used in this study. However, TRPV1 could be activated by endogenous ligands such as endocannabinoids or several lipoygenase metabolites as well as capsaicin intake.32,33 Especially, endocannabinoids are produced by stress-induced glucocorticoids in the brain.34 Therefore, TRPV1 activation could be achieved by lipid metabolites and stress-induced endocannabinoids at even lower concentrations of capsaicin that used in this study.

In summary, activation of TRPV1 and expression of HDAC2 in response to exogenous stimuli may constitute the molecular

Figure 4 Capsaicin reduces synapse density in mouse hippocampal primary neurons. (a) Targeting strategy for deleting HDAC2 mRNA. The mouse U6 promoter was used to drive the expression of HDAC2-shRNA (upper). Experimental design (lower). (b) Mouse Hdc2 and β-actin mRNA levels were quantified by qPCR (n=3–4 per group). Unpaired two-tailed t-test; *P<0.05 (c) Mouse Synapsin I and β-actin mRNA levels were quantified by qPCR (n=3 per group). Two-way analysis of variance (ANOVA; main effect of interaction F1,8=14.19, P=0.0055; virus F1,8=7.892, P=0.0229) followed by Bonferroni’s multiple comparison test. **P<0.01. (d) Triple-immunostaining of lenti-shLuc- or lenti-shHDAC2-infected neurons with anti-synapsin1, anti-MAP2 and anti-GFP antibodies. (e) Quantification of the number of synapsin1 (+) MAP2 (+) synaptic puncta per 10 μm of dendrite in lenti-shRNA-infected neurons (n=14–20). Two-way ANOVA (main effect of interaction F1,60=4.758, P=0.0331; virus F1,60=9.34, P=0.0033; drug F1,60=8.195, P=0.0058) followed by Bonferroni’s multiple comparison test. **P<0.01. Scale bar 5 μm. Values are means±s.e.m. GFP, green fluorescent protein; HDAC, histone deacetylase; qPCR, quantitative polymerase chain reaction; shRNA, short hairpin RNA.
mechanisms that together contribute to neuroplasticity. Our findings provide evidence that TRPV1 modulates HDAC2 binding to the promoters of synaptic molecules and suggest that epigenetic mechanisms play an important role in TRPV1-mediated neurodegeneration.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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