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Morphine and Fentanyl Citrate Induce Retrotransposition of Long Interspersed Element-1

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Abstract. *The retroelement long interspersed element-1 (LINE-1 or L1) comprises about 17% of the human genome. A single human cell has 80 to 100 copies of retrotransposition-competent L1, approximately 10% of which are 'hot' and actively 'jump' around the genome. Recent observations demonstrated that low-molecular weight compounds may induce L1 retrotransposition through unknown mechanisms. Herein, we demonstrated that the painkillers morphine and fentanyl citrate trigger L1 retrotransposition in neuronal cells without inducing DNA damage or up-regulating L1 mRNA expression. This effect was blocked by an antagonist of Toll-like receptor 4 (TLR4). Taken together, the data suggest that L1 retrotransposition due to morphine and fentanyl citrate is distinct from that triggered by DNA damage, requires TLR4, and is a novel type of genomic instability. Thus, we propose that L1 retrotransposition should be characterized as a component of the pharmacological activity of these analgesic agents.*

Long interspersed element-1 (*LINE-1* or *L1*), a highly active autonomous retrotransposon, is the most abundant endogenous retroelement in humans, and accounts for approximately 17% of the genome (1, 2). Notably, *L1* can retrotranspose not only itself but also other retroelements such as Alu and SVA (SINE-VNTR-*Alu*: short interspersed element-variable number tandem repeat-Alu) (3). A single human cell has more than 5×10^5 copies of L1, most of which are functionally silent (4). However, 80-100 copies are retrotransposition-competent (4), and approximately 10% of these are highly active and retrotranspose *via* target site-primed reverse transcription, a 'copy and paste' mechanism (5). Strikingly, the number of copies of *L1* is higher in human brain tissue (6, 7), and the retroelement is expressed and retrotransposed at high frequency in the mammalian nervous system (6-8). Thus, most recent studies of *L1* retrotransposition have focused on human neural stem cells (9). However, other studies have provided evidence that *L1* retrotransposition also occurs in somatic cells (6, 7), in which deregulation of the process may trigger various disease state due to gene deletions (10, 11), DNA damage (12), apoptosis, (13) and immune responses (14).

The opioid pain relievers morphine and fentanyl citrate are also used as decongestive agents in patients with cancer. However, these drugs also cause anxiety and dependence *via* mechanisms that are not clear. Recently, Wang *et al.* demonstrated that morphine triggers cell signaling *via* Toll-like receptor 4 (TLR4) (15), and propagates inflammatory signals in microglia. Indeed, TLR4 often induces inflammation after chronic drug abuse, although it is primarily a virus receptor.

Materials and Methods

Ethics statement. DNA recombination experiments were conducted with the approval of the Hyogo College of Medicine (No. 212001) and Meikai University School of Dentistry (No. 0104).

Chemicals and cells. Protease and phosphatase inhibitors were purchased from Roche Diagnostics (Basel, Switzerland). Morphine and fentanyl citrate were procured from Takeda Pharmaceutical Company (Osaka, Japan) and DAIICHI SANKYO Company (Tokyo, Japan) through a license (No. 3084) issued by the governor of Hyogo, Japan. TAK-242 TLR4 inhibitor was purchased from Millipore (Darmstadt, Germany), as were antibodies against H2AX and γ-H2AX. Anti-mouse and anti-rabbit secondary IgG conjugated to horseradish peroxidase were obtained from DAKO Japan (Tokyo, Japan). For immunohistochemical analysis, goat anti-mouse IgG conjugated to Alexa Fluor 555 (Life Technologies, Waltham, MA USA) was used as secondary antibody. Hoechst 33258 was also purchased from Life Technologies.

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SH-SY5Y cells (a human neuroblastoma cell line, EC94030304-F0), HuH-7 cells (a human hepatocellular carcinoma cell line, EC01042712-F0), and HT1080 cells (a human fibrosarcoma cell line; EC85111505-F0) were obtained from DS Pharma Biomedical (Osaka, Japan). SH-SY5Y cells were maintained at 37˚C and 5% CO_2 in Eagle's minimum essential medium/Ham's F12 medium supplemented with 1% non-essential amino acids and 15% fetal bovine serum (Hyclone, Thermo Scientific, Waltham, MA USA). HuH-7 and HT1080 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Transfection efficiencies were ~70% and ~30%, respectively, as determined by fluorescence-activated cell sorting 2 days after transfection of plasmid DNA encoding enhanced green fluorescent protein (data not shown).

L1 retrotransposition. L1 retrotransposition was assayed as described previously (16-20) using reporter vectors based on *pCEP4/L1mneoI/ColE1* (pL1-Neo^R) (10). Each construct contained all components of human L1 in a single transcriptional unit, with a neomycin-resistance gene in the reverse orientation. When *L1* retrotransposes, the intron within the neomycin (G418) resistance gene is spliced out, resulting in a neomycin-resistant cell. Constructs were transfected into cells using Lipofectamine 2000 (Life Technologies), with selection for 2 days on 50 μg/ml hygromycin. Transformants were then transferred to 100-mm plates at ~1×10^5 per plate, treated for an additional 2 or 3 days with opioids (morphine and fentanyl citrate at 0.1-40 μM) and control agents (HBS buffer), and finally grown on 270 μg/ml neomycin to select for cells in which retrotransposition occurred. After 3-4 weeks, colonies were stained with methylene blue, and counted.

Real-time reverse transcription polymerase chain reaction (RT-PCR). Real-time RT-PCR was performed essentially as described previously 16). Briefly, total RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany), and treated for 30 min at 25˚C with RNase-free DNase I (Qiagen). First-strand cDNA was prepared using random hexamers. Omniscript RT Kit (Qiagen) was used for reverse transcription, and targets were amplified and quantified using SYBR Premix Ex *Taq* (TaKaRa, Tokyo, Japan) on an Applied Biosystems StepOnePlus Real-Time PCR system (Life Technologies), following the manufacturers' instructions. Endogenous and exogenous *ORF2* was quantified using the primers L1-EGFP+5653F (5'-CCAAATGT CCAACAATGATAGACTG-3') and *L1-EGFP*+5762R (5'-CCATGTC CCTACAAAGGATATGAAC-3'). However, in order to correctly measure the abundance of mature L1 mRNA from pEF06R, it was necessary to exclude the effects of anti-sense mRNA transcribed from the inverted 3' *CMV* promoter (16). Mature *EGFP* was amplified with L1-EGFP+6342F (5'-TAGTGGTTGTCGGGCAGCAG-3') and L1-EGFP+7351R (5'-TTCAAGATCCGCCACAACATC-3'), while precursor *EGFP* was amplified with L1-EGFP+7222F (5'-TGGAAG CTGGGTGTGTAGTTATCTG-3') and /L1-EGFP+7365R (5' GGCAT CAAGGTGAACTTCAAGATC-3'). Expression was normalized to β-actin, which was quantified using 5'-GAGGG AAATCGTGCGTGA-3' and 5'-AGAAGGAAGGCTGG AAAA-3'. Data were collected from triplicate samples.

Western blotting. Cells were washed with phosphate-buffered saline (PBS), and resuspended in 50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.5% deoxycholic acid, 1% NP-40, and protease inhibitors. Cells were ultrasonicated for 12.5 min (10 s on, 20 s off) at medium power (250 W) and 4˚C in a Bioruptor (UCD-250; Cosmo Bio, Tokyo, Japan), and soluble cellular extracts were recovered after centrifugation for 10 min at 16,000 × *g*. Protein concentration was determined using the BCA Protein Assay Reagent Kit (Thermo Scientific Waltham, MA USA), and extracts were analyzed by western blotting. Blots were probed with primary antibodies to γ-H2AX and H2AX, labeled with secondary antibodies conjugated to horseradish peroxidase, and visualized using Pierce Western Blotting Substrate Plus (Thermo Scientific).

Immunostaining. Treated SH-SY5Y cells were washed with PBS, fixed with 4% paraformaldehyde in PBS, and permeabilised for 5 min with 0.5% Triton X-100 in PBS. Cells were then blocked for 30 min with 5% bovine serum albumin in PBS, probed with antibodies against γ-H2AX for 1 h at 37˚C, and labeled for 1 h at 37˚C with secondary antibodies conjugated to Alexa 555. Nuclei were stained with Hoechst 33258. Slides were mounted in anti-fade solution, and examined under a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan).

Statistical analysis. Statistical significance was evaluated using Mann–Whitney *U*-test, with n more than four. *p*-Values of less than 0.05 were considered statistically significant.

Results

Morphine and fentanyl citrate induce L1 retrotransposition. A colony-formation assay, in which an L1 transposition event is captured by expression of a neomycin resistance gene from the reporter construct *pCEP4/L1mneol/ColE1* (Figure 1A, pL1-Neo^R) was first established and validated using abuse drug, morphine or fentanyl citrate. Methamphetamine was previously shown to induce *L1* retrotransposition. Using this assay, we measured the ability of morphine and fentanyl citrate to induce *L1* retrotransposition in cells first transfected with the reporter construct. Transformants were selected on hygromycin, and subsequently treated with morphine or fentanyl citrate on day 3 after transfection (Figure 1B). Finally, cells were grown on neomycin to select for those in which retrotransposition occurred.

SH-SY5Y neuronal cells were treated with different concentrations of morphine and fentanyl citrate. Notably, 20 μM morphine and 10 μM fentanyl citrate induced retrotransposition to an extent comparable with that with 30-40 neomycin-resistant colonies in each plate (Figure 1C). Furthermore, we found morphine and fentanyl citrate to induce *L1* retrotransposition in a dose-dependent manner (Figure 1C-E). The frequency of retrotranspositions due to morphine and fentanyl citrate was approximately one per 2.5×10^3 SH-SY5Y cells (Figure 1D). In contrast, the opioids did not induce *L1* retrotransposition in HuH-7 cells (Figure 1F). Cells were treated for 2 days with morphine or fentanyl citrate at doses ranging from 40 μM to 2.5 μM, and then 500 cells of each sample were plated onto 6-cm plates. Cells were cultured for an additional 7 days, fixed, and stained with methylene blue. Cytotoxicity was not observed in SH-SY5Y cells (Figure 1G) or HuH-7 cells and HT1080 (data not shown) at concentrations sufficient to induce retrotransposition.

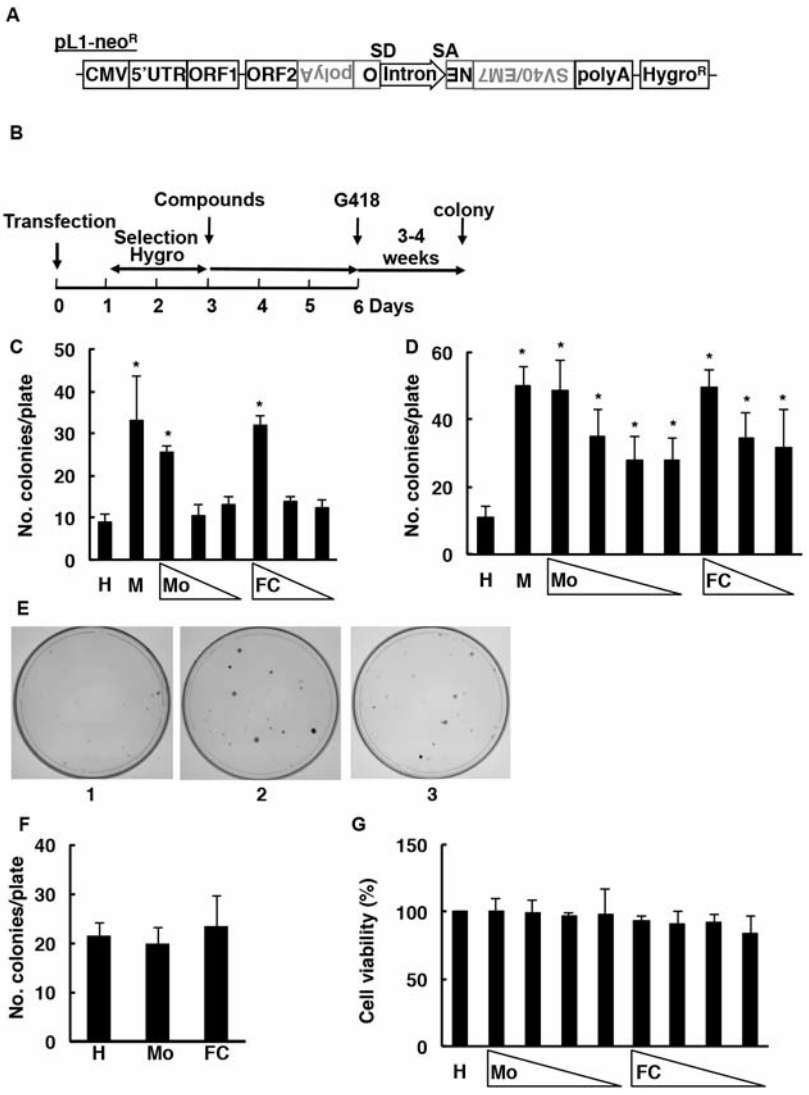

Figure 1. *Retroelement long interspersed element-1 (L1) retrotransposition due to morphine and fentanyl citrate in SH-SY5Y. A: Constructs used to measure L1 retrotransposition, whose details are provided in the Materials and Methods. B: Colony-formation assay. pL1-Neo^R was transfected into cells with selection for 2 days on hygromycin. Transformants were then exposed for 3 days to morphine or fentanyl citrate, and selected on G418 (neomycin) beginning on day 6. C: Colony-formation assay for L1 retrotranspositions in a neuroblast cell line as a result of exposure to HBS buffer (H), methamphetamine (M; 0.13 mM), morphine (Mo; 20, 2 and 0.2 μM), or fentanyl citrate (FC; 10, 1 and 0.1 μM). D: Dose-dependence of opioid-induced L1 retrotransposition in SH-SY5Y cells treated with HBS buffer (H), 0.13 mM methamphetamine (Me), morphine (Mo; 20, 10, 5, and 0.25 μM), or fentanyl citrate (FC; 10, 5, and 2.5 μM). E: Colonies formed after treatment in HBS buffer (plate 1), 20 μM morphine (plate 2), or 10 μM fentanyl citrate (plate 3). F: Morphine and fentanyl citrate did not induce L1 retrotranspositions in hepatocellular carcinoma cells exposed to HBS buffer (H), 20 μM morphine (Mo), and 10 μM fentanyl citrate (FC). G: Morphine and fentanyl citrate at doses sufficient to induce L1 retrotransposition were not cytotoxic to SH-SY5Y cells. HBS buffer (H); morphine (Mo; 40, 20, 10, and 5 μM); fentanyl citrate (FC; 20, 10, 5, and 2.5 μM). At least two independent experiments were performed, and representative results are shown. The mean numbers of colonies ± S.D. are shown. Asterisks indicate statistical significance ($p<0.01$ compared with HBS buffer).*

Morphine and fentanyl citrate do not up-regulate L1 mRNA. We investigated the possibility that morphine and fentanyl citrate induce retrotransposition in SH-SY5Y cells by up-regulating expression of *L1*. To measure *L1* expression, we used *pEF06R*, a reporter construct that contains *L1* fused to enhanced *EGFP* (Figure 2A) (16). However, to accurately measure the abundance of mature *L1* mRNA, it was necessary to exclude the effects of anti-sense mRNA transcribed from the inverted 3' *CMV* promoter (16). Quantitative real-time RT-PCR indicated that morphine and

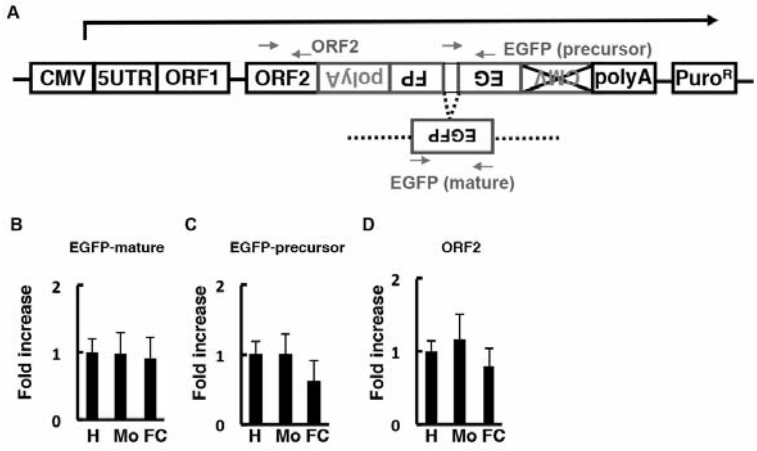

Figure 2. *Morphine and fentanyl citrate did not increase expression of retroelement long interspersed element-1 (L1). A: Primers used for quantitative real-time polymerase chain reaction, and a construct without a 3' CMV promoter region (pEF06RΔ3'CMV), that was used to exclude the effects of anti-sense EGFP mRNA. B, C: Morphine and fentanyl citrate did not affect expression or splicing of precursor L1 mRNA. In addition, the opioids did not affect expression of mature and precursor EGFP mRNA in SH-SY5Y cells transfected with pEF06RΔ3'CMV. D: Exposure for 24 h to morphine and fentanyl citrate did not induce expression of endogenous ORF2. H, HBS buffer control; Mo, 20 μM morphine; FC, 10 μM fentanyl citrate.*

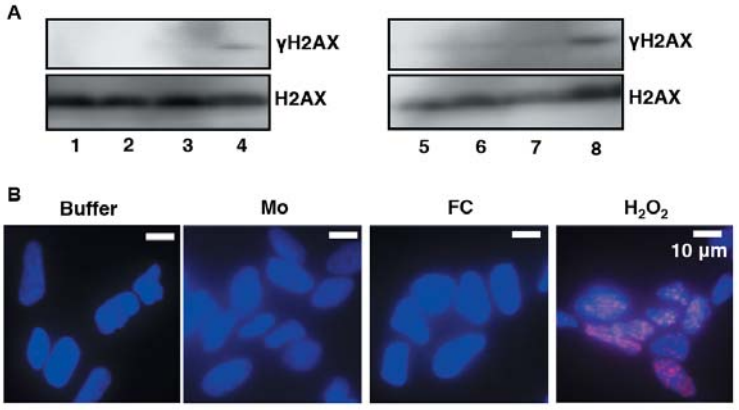

Figure 3. *Morphine and fentanyl citrate do not induce DNA damage. A: Morphine and fentanyl citrate did not induce expression of phosphorylated H2AX, a marker of DNA damage. Lane 1, HBS buffer; lanes 2-3, 20 and 10 μM morphine; lanes 6-7, 10 and 5 μM fentanyl citrate; lanes 4 and 8, 1 mM H_2O_2. B: Immunohistochemical analysis for phosphorylated H2AX in SH-SY5Y cells treated for 1 day with HBS buffer (Buffer), 20 μM morphine (Mo), 10 μM fentanyl citrate (FC), and 1 mM H_2O_2. Scale bar, 10 μm.*

fentanyl citrate did not increase the splicing efficiency of the immature *L1* transcript (Figure 2B and C) nor of *ORF2* mRNA (Figure 2D).

Morphine and fentanyl citrate do not induce DNA double-strand breaks. Farkash *et al*. reported that DNA double-strand breaks induced by gamma radiation increase *L1* retrotransposition (21). Hence, we tested whether morphine and fentanyl citrate stimulate retrotransposition by inducing double-strand breaks. SH-SY5Y cells treated with 20 and 10 μM morphine or 10 and 5 μM fentanyl citrate were analyzed by western blot for expression of H2AX phosphorylated at serine 139 (γ-H2AX), a marker of DNA damage. The drugs did not induce expression of γ-H2AX (Figure 3A), and did not generate γ-H2AX foci even at high doses in contrast to the positive control (H_2O_2) (Figure 3B). These observations indicate that retrotransposition events due to morphine and fentanyl citrate are attributable to non-genotoxic effects.

L1 retrotransposition due to opioids depends on TLR4. Because morphine and fentanyl citrate have been reported to trigger TLR4 signaling (15), we investigated whether the receptor is involved in opioid-induced *L1* retrotransposition. The data demonstrate that TAK-242, an inhibitor of TLR4, also inhibited *L1* retrotransposition, suggesting the involvement of TLR4 for *L1* retrotransposition (Figure 4).

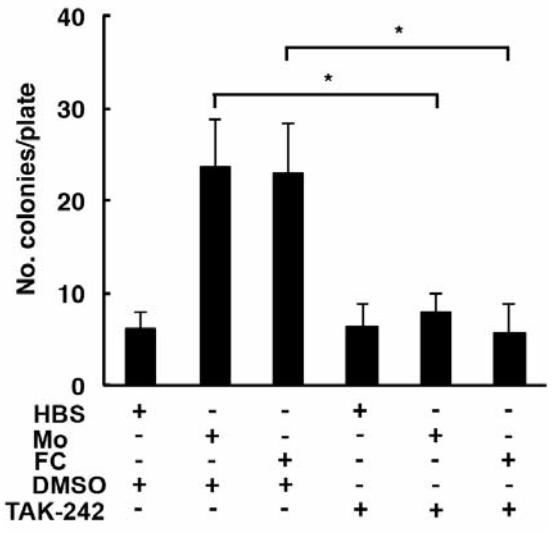

Figure 4. *Effect of a Toll-like receptor 4 (TLR4) antagonist on opioid-induced retroelement long interspersed element-1 (L1) retrotransposition. Cells were treated with 1 μM TAK-242 for 1 h prior to treatment with 20 μM morphine or 10 μM fentanyl citrate. The mean numbers of colonies ± S.D. are shown. Asterisks indicate statistical significance p<0.01.*

Discussion

Morphine and fentanyl citrate are used to manage sharp pain during medical procedures, but dependence is a serious risk. However, the detailed molecular and genomic bases of dependence have not been characterized. In this study, we demonstrated that morphine and fentanyl citrate induce *L1* retrotransposition in SH-SY5Y neuronal cells, but not in HuH-7 cells. Notably, we found that retrotransposition due to these drugs is distinct from retrotransposition due to double-strand breaks, as indicated by the inability of morphine and fentanyl citrate to boost expression of γ-H2AX (22), a sensitive marker of such breaks. Our results significantly expand the repertoire of small molecules that destabilize the genome by inducing retrotransposition, which includes tryptophan photoproducts (16) and environmental carcinogens (23). Moreover, TLR4 appears to be required, as shown by the ability of the TLR4 inhibitor TAK-242 to block retrotransposition (24). However, the mechanistic details by which opioids activate TLR4 remain unknown (15), as the receptor is typically stimulated by viral and inflammatory stimuli.

Recent evidence shows that *L1* retrotranspositions are highly frequent in neurons (7), and that L1 expression in neuronal stem cells increases in response to environmental changes (6), especially in the hippocampus (25). These reports, along with our results, suggest that drug abuse and depression may induce *L1* retrotranspositions in the brain and spinal cord (8, 9, 25). Taken together, we believe that *L1* retrotransposition due to opioids may deregulate TLR4 signaling in neurons (20).

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