**Supplemental Digital Content 2**

**Measurement of mitochondrial membrane potential (Δψm) using two different dyes**

**A. Measurement of mitochondrial membrane potential (Δψm) in intact cells using the dye JC-1**

For these experiments, cells were plated and grown in three 96-well microplates in replicates (at least 24 replicates for each treatment per plate (24 contols, 24 fentanyl-treated and 24 5-HD + fentanyl-treated wells/plate) (n is at least 72 per condition). After incubation, mitochondrial electrochemical potential gradient (Δψm) in intact cells was measured using the cationic dye 5,5´,6´,6´-tetrachloro-1,1´,3,3´-tetraethylbenzimidazolocarbocyanine iodide (JC-1). JC-1 is a mitochondrial sensor which aggregates in polarized mitochondria, where it forms red fluorescent aggregates. Dissipation of the mitochondrial membrane potential prevents the accumulation of the JC-1 dye in the mitochondria, and the dye is dispersed throughout the entire cell, leading to a shift from red (JC-1 aggregates) to green fluorescence (JC-1 monomers). Thus the loss of JC-1 aggregates directly correlates with changes in Δψm. Briefly, for these experiments, the cells were grown in 96-well plates and treated with fentanyl for 1 hour (2 ng/ml), or pretreated with 5-HD at 50 µM for 30 minutes, followed by incubation with fentanyl at 2 ng/ml for an additional hour and the mitochondrial membrane potential was measured using the JC-1 mitochondria staining kit for mitochondrial potential change detection (Sigma, Switzerland) according to the manufacturer’s instructions. Δψm was measured immediately by fluormetry on a Tecan Infinite M1000 plate reader (Tecan, Männedorf, Switzerland). For JC-1 monomers, the fluorimeter was set at a 490 nm excitation wavelength and 530 nm emission wavelength and fluorescence was measured. For JC-1 aggregates, the fluorimeter was set at a 525 nm excitation wavelength and 590 nm emission wavelength and fluorescence was measured. Afterwards, the Δψm (590/530 nm fluorescence ratio) was calculated.

**B. Measurement of Δψm in intact cells using tetramethylrhodamine methyl ester (TMRM)**

For these experiments, cells were plated and grown in three 96-well microplates in replicates (18 replicates for each treatment per plate (18 contols, 18 fentanyl-treated, 18 fentanyl + 5-HD-treated and 18 5-HD-treated wells/plate) (n is 54 per condition). After incubation, mitochondrial electrochemical potential gradient in intact cells was measured using the lipophilic but slightly cationic fluorescent dye TMRM. The lipophilic structures of this dye allow it to easily penetrate cell and mitochondrial lipid bilayer membrane barriers. Briefly, for these experiments, the cells were grown in 96-well plates and treated with fentanyl for 1 hour (2 ng/ml) and the mitochondrial membrane potential was measured using the TMRM mitochondria staining kit for mitochondrial potential change detection (Immunochemistry Technologies, LLC, Bloomington, MN, USA) according to the manufacturer’s instructions. Cells with polarized mitochondria fluoresce bright red (the fluorimeter was set at a 540 nm excitation wavelength and 575 nm emission wavelength); cells with membrane-compromised cells exhibit a diminished fluorescence or no signal. Mitochondrial membrane potential levels were expressed as relative fluorescent units (RFU) per µg cellular protein.