**Supplemental Digital Content 3**

**Measurement of the HepG2 cells ATP content**

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 [1 hour (2 ng/ml)] and 18 5-HD + fentanyl-treated [5-HD at 50 µM for 30 minutes, followed by incubation with fentanyl at 2 ng/ml for an additional hour] wells/plate) (n is 54 per condition). In an additional series of dose-response experiments, cells were plated and grown in two 96-well microplates in replicates (12 replicates for each treatment per plate: 12 controls, 12 fentanyl-treated [1 hour (0.5 ng/ml)], 12 fentanyl-treated [1 hour (2 ng/ml)], 12 fentanyl-treated [1 hour (10 ng/ml)] and 12 fentanyl-treated [1 hour (50 ng/ml)] (n is 24 per condition). After treatment, cells were lysed directly in the 96-well plates and exposed to the ATP substrate solution using the Luminescent ATP Detection Assay Kit (Abcam, Cambridge, USA) according to the manufacturer’s instructions and signal was measured on a luminescent counter (Tecan Infinite M1000 plate reader, Tecan, Männedorf, Switzerland). The kit contains: detergent for cell lysis, substrate buffer, and lyophilized ATP standard. The assay kit is a cellular ATP monitoring system based on firefly (Photinus pyralis) luciferase and is based on the production of light caused by the reaction of ATP with added luciferase and D-luciferin. The emitted light is proportional to the ATP concentration inside the cell. Blank wells (without cells) were included to determine the background luminescence.

**Measurement of the HepG2 cells ADP/ATP ratio**

For these experiments, cells were plated and grown in one 96-well microplates in replicates (20 replicates for each treatment (20 controls and 18 fentanyl-treated [2 ng/ml fentanyl for 1 hour wells]. The ADP/ATP ratio was determined using the Bioluminescent ADP/ATP Ratio Assay Kit (Abcam, Cambridge, UK) according to the manufacturer’s instructions. The assay is based on the formation of ADP from ATP and luceferin, catalyzed by luciferase. The emitted light is measured on a luminescent counter. Afterwards, cellular ADP content is measured by its conversion to ATP and measured using the same reaction. Briefley, 100 μl of the reaction mix containing nucleotide releasing buffer and ATP monitoring enzyme was added to the appropriate wells of an empty 96-well luminometer plate and the background luminescence was measured (Data A). After treatment of the cells with fentanyl, the culture medium was removed and cells were treated at room temperature with 50 µl of the nucleotide releasing buffer. The released nucleotides were transferred to the 96-well luminometer plate and the luminescence was measured after 2 minutes (Data B). To determine cellular ADP levels content the samples were read again (Data C) and afterwards 1 μl of ADP converting enzyme was added into each well and the luminescence was measured after 2 minutes again (Data D). ADP/ATP content is calculated as (Data D – Data C)/(Data B – Data A).

**Measurement of the HepG2 cells FAD levels**

The cells were grown in 16 separate 25 cm2 culture flasks, and afterwards cells in 8 flasks were treated with vehicle alone and 8 flasks with fentanyl (n=8 for each group). FAD levels were measured using FAD assay kit (BioVision, Mountain View, CA, USA) according to the manufacturer’s instructions. In brief, cells were lysed and deproteinized. Unknown samples and FAD standard were mixed with FAD enzyme, OxiRed Probe and assay buffer for 15–60 min and measured by the colorimetric method (Absorbance: 570 nm). The data were expressed as µM/µg cellular protein and expressed as as the ratio (%) of fentanyl-treated cells versus untreated cells.

**Measurement of the HepG2 cells mitochondrial ATP synthase enzymatic activity (Complex V or F1F0 ATPase)**

For these experiments, the cells were grown in 36 seperate 25 cm2 cell culture flasks and treated placebo (n=12) with fentanyl for 1 hour (2 ng/ml) (n=12), or pretreated with 5-HD at 50 µM for 30 minutes, followed by incubation with fentanyl at 2 ng/ml for an additional hour (n=12). After treatment, cells were lysed, and detection of ATP synthase (oligomycin sensitive ATP hydrolytic) activity of whole cell extracts were performed using a 96-well plate–based assay from Mitosciences (Abcam, Cambridge, UK) according to the manufacturer’s instructions. The kit contains: buffers, detergent, reagent mix, lipid mix and a 96-well pre-coated with capture antibodies microplate (12 strips). The ATP synthase complex can work in reverse as a proton pumping ATPase and the assay goes in reverse. Briefly, cells were trypsinised, centrifuged and resuspended in the supplied buffer. The samples were then frozen, thawed and pelleted by centrifugation at 10,000 g. Once pelleted, the samples were resuspended again in the supplied buffer and protein concentrations were determined with the Quant-iTM assay kit and read with the Qubit-TM fluorometer (Invitrogen®, Basel, Switzerland). The samples were then extracted by adding 1/10 volume of the supplied detergent for 30 min on ice. Afterwards the samples were centrifuged at 10,000 g for 20 min and the resulting supernatants (50 µl sample volume) were loaded into individual wells of the supplied 96-well plate and incubated for 3 hours at room temperature. The kit is an in vitro enzyme-linked immunosorbent assay (ELISA) which employs a capture antibody specific for human ATP synthase coated onto microplate well strips. Afterwards the wells were rinsed and supplied lipid and reagent mix were added. The ATP hydrolytic activity is coupled to the molar conversion of NADH to NAD+ measured as a decrease in absorbance at OD 340 nm (for 1 hour at 30°C) and was oligomycin sensitive: >90%. The absorbance was measured with a VERSAmax microplate reader (Molecular Devices, Orleans Drive Sunnyvale, CA, USA) using a kinetic program with 1 min intervals between readings. Blank wells (buffer controls) were included to determine the background absorbance. The activity rate (the change in absorbance at 340 nm/minute) is expressed as mM/min/mg cellular extract.

**Determination of Release of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS)**

HepG2 cells were grown in a 96-well microplates and treated with fentanyl (2 ng/ml) for 1 hour (n=24 each: 24 controls and 24 Fentanyl-treated). Total free radicals (ROS and RNS) levels were measured in the cellular supernatants using the OxiSelect In Vitro ROS/RNS Assay Kit (Cell Biolabs Inc., San Diego, CA, USA). ROS and RNS species react with DCFH to the fluorescent 2’,7’-dichlorodihydrofluorescein (DCF). The free radical content in unknown samples is determined by comparison with the predetermined DCF standard curve.DCF fluorescence intensity is proportional to the total ROS and RNS levels within the sample and was measured by fluorimetry. The data is expressed as pmol/µg cellular protein and presented as the ratio (%) of fentanyl-treated cells versus untreated cells.

**Determination of mitochondrial calcium levels with Rhod 2-AM**

Mitochondrial calcium levels were measured using Rhod-2-AM. Rhod 2-AM is a membrane permeable form of Rhod 2 which is a calcium indicator molecule that exhibits an increase in fluorescence upon binding calcium. It is hydrolyzed in the cytosol and its net charge causes it to accumulate within mitochondria. For these experiments, the cells were plated and grown in a 96-well microplate in replicates (32 controls and 16 fentanyl-treated). and resuspended in RPMI1640 without phenol red and treated with fentanyl (2 ng/ml) for 1 hour. The cells were then incubated with 5 μM Rhod2-AM (Invitrogen, Carlsbad, CA, USA) for 30 min and washed two times with PBS. The changes in the fluorescence of the Rhod2-AM dye (λexc = 540 nm; λem = 590 nm) was measured in a microplate reader. Mitochondrial calcium levels were expressed as relative fluorescent units (RFU) per µg cellular protein.