

Opioid Antagonist Nanodrugs Successfully Attenuate the Severity of Ischemic Stroke

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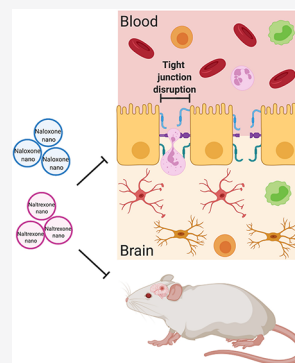
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ABSTRACT: The United States is in the midst of an opioid epidemic that is linked to a number of serious health issues, including an increase in cerebrovascular events, namely, stroke. Chronic prescription opioid use exacerbates the risk and severity of ischemic stroke, contributing to stroke as the fifth overall cause of death in the United States and costing the US health care system over \$30 billion annually. Pathologically, opioids challenge the integrity of the blood–brain barrier (BBB), resulting in a dysregulation of tight junction (TJ) proteins that are crucial in maintaining barrier homeostasis. Despite this, treatment options for ischemic stroke are limited, and there are no pharmacological options to attenuate TJ damage, including in incidents that are linked to opioid use. Herein, we have generated carrier-free, pure “nanodrugs” or nanoparticles of naloxone and naltrexone with enhanced therapeutic properties compared to the original (parent) drugs. The generated nanoformulations of both opioid antagonists exhibited successful attenuation of morphine- or oxycodone-induced alterations of TJ protein expression and reduced oxidative stress to a greater extent than the parent drugs (non-nano). As a proof of concept, we then proceeded to evaluate the therapeutic effectiveness of the generated nanodrugs in an ischemic stroke model of mice exposed to morphine or oxycodone. Our results demonstrate that the opioid antagonist nanoformulations reduced stroke severity in mice. Overall, this research implements advances in nanotechnology-based repurposing of FDA-approved therapeutics, and the obtained results also suggest underlying pharmacological mechanisms of opioid antagonists, further supporting these opioid antagonists and their respective nanoformulations as potential therapeutic agents for ischemic stroke.



KEYWORDS: ischemic stroke, therapeutic, opioid antagonists, naloxone, naltrexone, nanotechnology

INTRODUCTION

Since 2017, the United States has been battling a public health emergency—the opioid epidemic, with over 2 million people suffering from an opioid use disorder.¹ Prescription opioid use, such as long-term use of morphine and oxycodone has become the predominant treatment for acute and chronic pain; however, opioid use may also be voluntary for recreational purposes. Subsequently, cases of opioid abuse, as well as opioid-related cerebrovascular complications, namely, ischemic stroke, have become highly prevalent.^{2–6} While chronic prescription opioid use is linked to a higher risk for stroke, currently there are no approved pharmacological agents for the resulting pathological damage to the tight junction (TJ) proteins of the blood–brain barrier (BBB) that arises from an ischemic stroke. Additionally, there is a need for novel drugs to improve stroke outcomes as there are no approved neuroprotective or neurorestorative treatments for stroke. Because BBB disruption is a pathological hallmark in ischemic stroke, protection of the TJ proteins as a therapeutic strategy during stroke and for stroke recovery is of critical importance.⁷

An ischemic stroke accounts for 87% of all strokes and occurs when there is an obstruction in the blood vessel, such as a blood clot, restricting blood flow into the brain parenchyma.⁵

During an ischemic stroke, the affected area suffers oxidative stress, in turn challenging the integrity of the BBB and resulting in its breakdown, ultimately leading to neuronal dysfunction, neuroinflammation, and neurodegeneration.^{8–11} Much of the vascular and tissue damage in stroke is attributed to neuroinflammation and oxidative stress, with oxidative stress as one of the underlying causes of BBB disruption in ischemic stroke.^{8,10–12} Dysregulation of TJ proteins such as occludin, junctional adhesion molecule, and zonula occludens is observed in ischemic stroke.^{12,13} As TJ proteins are crucial to the integrity of the BBB, alterations of their expression can promote the migration and activation of inflammatory cells across the BBB, resulting in neuroinflammation.

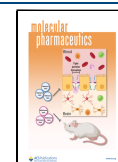
Pain management by prescription opioids is critical in the effective care of patients after surgery, as well as patients with cancer and severe acute and chronic diseases.^{14,15} Moreover,

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opioid misuse and addiction may originate recreationally. Opioids are a class of drugs naturally found in the opium poppy plant and work in the brain to produce a variety of effects, including pain relief.¹⁶ Commonly prescribed opioids, such as morphine, oxycodone, hydrocodone, codeine, and methadone, have been a basis of pain treatment regimens.^{17–19} They block pain signals between the brain and the body and are typically prescribed to treat moderate to severe pain.²⁰ In addition to controlling pain, opioids induce feelings of relaxation and euphoria and are highly addictive. Side effects can include slow breathing, constipation, nausea, confusion, and drowsiness.²¹

Pathologically, opioids challenge the integrity of the BBB, which is a critical regulatory interface that maintains homeostasis between the peripheral circulation and central nervous system (CNS).^{22–25} Cerebral endothelial cells, along with astrocytes, pericytes, neurons, and microglia, constitute the basic neurovascular units of the BBB.¹² The endothelial cell–cell interactions are mediated by junctional complexes known as TJs,²⁶ which are essential gate-keeper proteins of the BBB to prevent diffusion of blood-borne substances to the brain. Subsequently, disruption of TJ proteins may lead to further neuronal dysfunction, neuroinflammation, and neurodegeneration.^{8–11}

Chronic prescription opioid use induces mitochondrial dysfunction and oxidative stress, which are critical factors in stimulating neuroimmune activation. As a result, opioids are linked to a higher risk for stroke by restricting blood flow through the carotid artery or causing cardio-embolism, hypoxia, or hypoperfusion.^{2,27,28} Currently, there is only one FDA-approved drug for stroke treatment: tissue-type plasminogen activator (tPA), which has no apparent neuroprotective or neurological recovery effects.²⁹ To address this deficiency, we propose a novel repurposing and formulation of opioid antagonists, naloxone and naltrexone, into “nanodrugs” or nanoparticles as therapeutic agents for ischemic stroke. Naloxone and naltrexone are FDA-approved opioid antagonists currently used for opioid abuse treatment.^{5,30–32} The anti-inflammatory properties of opioid antagonists and potential minimization of TJ disruptions make these drugs attractive potential stroke therapeutics.⁷ In order to potentially enhance the design of opioid antagonists for stroke therapeutics, the current study implements novel strategies and advances in the field of nanotechnology and nanoparticle synthesis for efficient stroke therapeutics. Nanotechnology is an emerging drug development strategy that preserves the innate therapeutic and non-toxic properties of the original drug while optimizing the shape and size of the drug molecule to achieve a “nanodrug” or nanoparticle formulation for increased therapeutic efficacy.³³ The flexibility in controlling the shape and size of the nanoparticles allows for tailoring to achieve a very small size for targeting and enhancing delivery to the location of interest, like the BBB.^{34,35} To date, nanoparticles of opioid antagonists have been synthesized using polymer or copolymer methods.^{36,37} No reports of nontoxic, carrier-free pure nanodrugs of these opioid antagonists have been reported or tested in vitro and in vivo, specifically in the context of stroke and opioid abuse. Herein, we formulate carrier-free nanodrugs or nanoparticles of naloxone and naltrexone and evaluate them for therapeutic efficacy. Carrier-free nanoformulations of the drug will improve the loading capacity of drug and avoids issue with the toxicity or metabolism of the carrier or polymer in the body.³⁸

In the current study, we evaluate the potential therapeutic efficacy of naloxone and naltrexone and their respective generated nanoformulations, in vitro and in vivo, in the context of opioid-induced stroke. We also seek further understanding of the underlying pharmacological mechanisms, with the focus on the modulation of the NADPH oxidase and TLR4 signaling, attributing to the neuroprotective effects of opioid antagonists. Our focus on the impact of opioids on TJ proteins, as well as a novel repurposing and formulation of naloxone and naltrexone nanodrugs to treat TJ dysfunction and stroke is unexplored. We seek to generate new knowledge regarding opioid-induced cellular alteration in the neurovascular unit and cellular and molecular mechanisms underlying damage to the cerebrovascular system by chronic opioid exposure, ultimately, to provide information about potential novel opioid antagonist therapeutics and to target and reduce TJ protein dysregulation and stroke severity.

■ METHODS

Cell Cultures and Opioid Drug Administration. PC12 (ATCC CRL-1721) cells were cultured on Type IV collagenized Corning cell culture flasks (surface area 25 cm², canted neck) at 5% CO₂, 37 °C according to the American Tissue Culture Collection (ATCC) protocol. Complete culture medium was prepared using RPMI 1640 with 10% heat inactivated horse serum, 5% fetal bovine serum, and 50 units/50 μg per mL Penn/Strep/Glutamine (each). Cell culture medium was changed three times a week. Cells were washed with phosphate-buffered saline (PBS) prior to sub-culturing using 0.25% trypsin/EDTA. Once confluent, cells were sub-cultured to a Type IV collagenized 96-well plate for the cell viability assay.

hCMEC/D3 BBB cell line (Sigma-Aldrich SCC066) was cultured and maintained according to the protocol. Cells were maintained in pre-collagenized vented cap Corning cell culture T75 flasks (surface area 75 cm², canted neck). Flasks and plates were collagenized using a 1:50 rat Collagen Type 1 rat tail (Corning 354236) to UltraPure DNase/RNase-free distilled water (Fisher Scientific Invitrogen 10,977,015). Cell culture medium was changed three times a week with EBM-2 Endothelial Cell Growth Basal Medium-2 mixed with growth factors and supplements (EGM-2 MV Microvascular Endothelial SingleQuots Kit CC-4147). Once 80–90% T75 flask confluency was reached, cells were sub-cultured to pre-collagenized 6-well and 96-well plates.

Morphine sulfate (NIDA 9300-001) and oxycodone hydrochloride (Sigma-Aldrich O1378) were dissolved in PBS and used for cell treatment at the concentrations of 100 and 25 μM, respectively.

Nanodrug Preparation and Characterization. Pure drug, nontoxic, carrier-free naloxone and naltrexone nanodrugs were prepared using a bottom-up reprecipitation method in which organic molecules interact with one another to form an aggregate.³⁹ The reprecipitation method involves dissolving a small amount of organic material in a good solvent, in the excess of a poor solvent to form pure nanodrug precipitates, without the need of a polymer of the lipid-based carrier.³⁹ A major challenge for delivering drugs that target the CNS is the inability for most drugs to cross the BBB and enter brain tissue.^{40,41} Since it is known that nanoparticles prepared with nonionic surfactants exhibit increased uptake by the brain, we employed Tween 20 during nanodrug formulations for more successful passage into the BBB.^{34,40,41} Pure powdered

naloxone [United States Pharmacopeia (USP) Reference Standard 1453005] was dissolved in ethyl alcohol to make a stock solution of 0.05 M. Then, 50 μ L of naloxone stock was added dropwise to 0.1% Tween 20 under stirring in the glass vial, covered with a parafilm, and left to stir for 24 h to allow for nanoparticle formation. The parafilm was punctured to allow ethanol release. Naltrexone nanoparticles were synthesized using a similar method. A starting stock solution of 0.16 M was made using pure powdered naltrexone (USP Reference Standard 1453504). Characterization of nanodrugs was performed by dynamic light scattering (DLS)⁴² and zeta potential (ζ -potential) using a Zeta sizer Nanoseries (Nano ZS90, Malvern, UK). Transmission electron microscopy (TEM) was conducted using a JEM-1400 TEM instrument (JEOL, München, Germany) and carbon-coated 400-mesh TEM grids (Ted Pella, Redding, CA).⁴³ UV spectra were recorded using a DS-11 spectrophotometer (DeNovix, DE, USA).

Nanodrug Cytotoxicity. Naloxone and naltrexone nanodrug formulations were evaluated for cytotoxicity by performing *in vitro* cell viability experiments in PC-12 cultures. PC-12 (ATCC CRL-1721) is a cell line originating from rat pheochromocytoma, which is widely used in neurotoxicological studies. In order to test cell viability and assess the risk of cellular toxicity, a range of naloxone and naltrexone nanodrug concentrations were added that encompass concentrations above and below the current FDA-approved dosages that are prescribed to patients, warranting flexibility for testing nanodrugs *in vitro*.⁴⁴

Immunoblotting. Naloxone and naltrexone nanoformulations were evaluated for attenuating endothelial TJ protein dysregulation through the assessment of TJ protein expression. The main cellular model system was the primary human brain microvascular endothelial cell line (hCMEC/D3). hCMECs were exposed to various concentrations of prescription opioids (morphine and oxycodone) twice a day for 24 h, followed by the assessment of TJ protein (occludin and claudin-5) expression by Western blot. The choice of TJ proteins was driven by the fact that occludin and claudin-5 are transmembrane proteins involved in the regulation of membrane integrity. Control experiments included treatment with vehicle, instead of opioid receptor antagonists.

hCMECs were washed with PBS and lysed using radioimmunoprecipitation assay (RIPA) lysis and extraction buffer (Thermo Scientific 89900) supplemented with a phosphatase/protease inhibitor cocktail (Cell Signaling 5872S). Cells from each well of a 6-well plate were individually scraped into microcentrifuge tubes and centrifuged at 4 °C at 12,000 RCF for 20 min. Protein concentration was assessed according to the protocol using the Pierce BCA Protein Assay Kit (Thermo Scientific 23225). Samples for loading gels were prepared according to concentrations calculated based on the BCA assay. Samples were individually denatured using Laemmli (6 \times , SDS-Sample Buffer, Boston BioProducts BP-111R) and placed on dry heat blocks (98 °C) for 5 min. Samples were separated on a 4–20% Mini-PROTEAN TGX Stain-Free Protein Gel (Bio-Rad Laboratories 4568094) and electrotransferred onto a polyvinylidene difluoride (PVDF) membrane using a Trans-Blot Turbo Transfer System (Bio-Rad Laboratories 170-4159). Blots were blocked with 5% bovine serum albumin⁴⁵ (Sigma-Aldrich A7906-500G) for 1 h and incubated overnight at 4 °C with primary antibodies used in the following ratios: occludin (Thermo Fisher Scientific 33-

1500, 1:500), claudin-5 (Thermo Fisher Scientific 34-1600, 1:1000), and GAPDH (NOVUS NB600-5021R, 1:10,000) in 5% of BSA-Tris-buffered saline (TBS). The following day, blots were washed three times with TBS-0.05% of Tween 20 for 5 min and incubated for 1 h with secondary antibody (1:10000) (LI-COR 926-32210 926-32211) in 5% of BSA-TBS. Blots were once again washed with TBS-0.05% of Tween 20 three times and visualized by the Licor CLX imaging system. Image Studio 4.0 software (LI-COR) was used for signal quantification.

Superoxide Assessment. hCMEC/D3 cells were seeded at a density of 10,000 cells/well in 96-well clear bottom plates and grown to confluency. Dihydroethidium (DHE) Assay Kit – Reactive Oxygen Species (ab236206) was used to directly measure superoxide generation. Assays were conducted according to the kit protocol, and DHE fluorescence was measured using an excitation wavelength of 500 nm and an emission wavelength of 585 nm. NSC 23766 (100 μ M) (Tocris 2161) was used to inhibit the NADPH complex, and TAK 242 (100 μ M) (Tocris 6587) was used for TLR4 inhibition.⁴⁷

Animals, Treatment Regimen, and the Middle Cerebral Artery Occlusion Surgery.

All animal procedures were approved by the University of Miami Institutional Animal Care and Use Committee and performed in accordance with the relevant guidelines and regulations (IACUC 21-020). Male C57BL/6J mice (Jackson Laboratories) with 14 weeks of age were allowed to acclimatize to the animal facility for 1 week with free access to food and water. Mice were weight-matched and randomly assigned to different treatment groups. Opioids were administered with two repeating doses in 1 day to reflect a pattern of acute exposure of drug abusers. Mice were *i.p.* injected with morphine sulfate (80 mg/kg, National Institute on Drug Abuse) or oxycodone chloride (40 mg/kg, Sigma Aldrich) dissolved in saline, twice during a 10 h interval. Control mice were injected with saline as a vehicle. We determined the dosages of naloxone and naltrexone in mice based on the extrapolation from the clinic dosages in humans.⁴⁸ For mice treated with naloxone nanoparticles or naltrexone nanoparticles, one dose of nanoformulation loaded with naloxone (0.65 mg/kg) or naltrexone (2.19 mg/kg) was given through *i.v.* injection 30 min after each morphine or oxycodone dose. When naloxone or naltrexone (parent drugs) was used, one dose of naloxone (0.65 mg/kg) or naltrexone (2.19 mg/kg) was given through *i.p.* injection 30 min after each morphine or oxycodone dose. It is important to note for drugs administered by intraperitoneal injection that molecular size would influence the absorption pathways from the peritoneal cavity to the systemic circulation and minimally affect the overall absorption of pharmacological agents. For the nanoparticle formulations, the absorption rate and bioavailability of the administered drug would be largely affected by the physicochemical properties of the drug, dissolution rate of the suspension, and particle size.⁴⁹ To avoid the possible impact on the bioavailability of the nanomedicines, we chose to administer the nanomedicines by intravenous injection, instead of intraperitoneal injection for small-molecule drugs. Afterward, mice were used for the ischemic stroke procedure.

An ischemic stroke was induced at 24 h after the first opioid dose by the middle cerebral artery occlusion (MCAO) as previously described.¹⁰ Briefly, occlusion was performed by inserting a silicone-coated suture (Doccol) into the common carotid artery and blocking blood flow to the middle cerebral

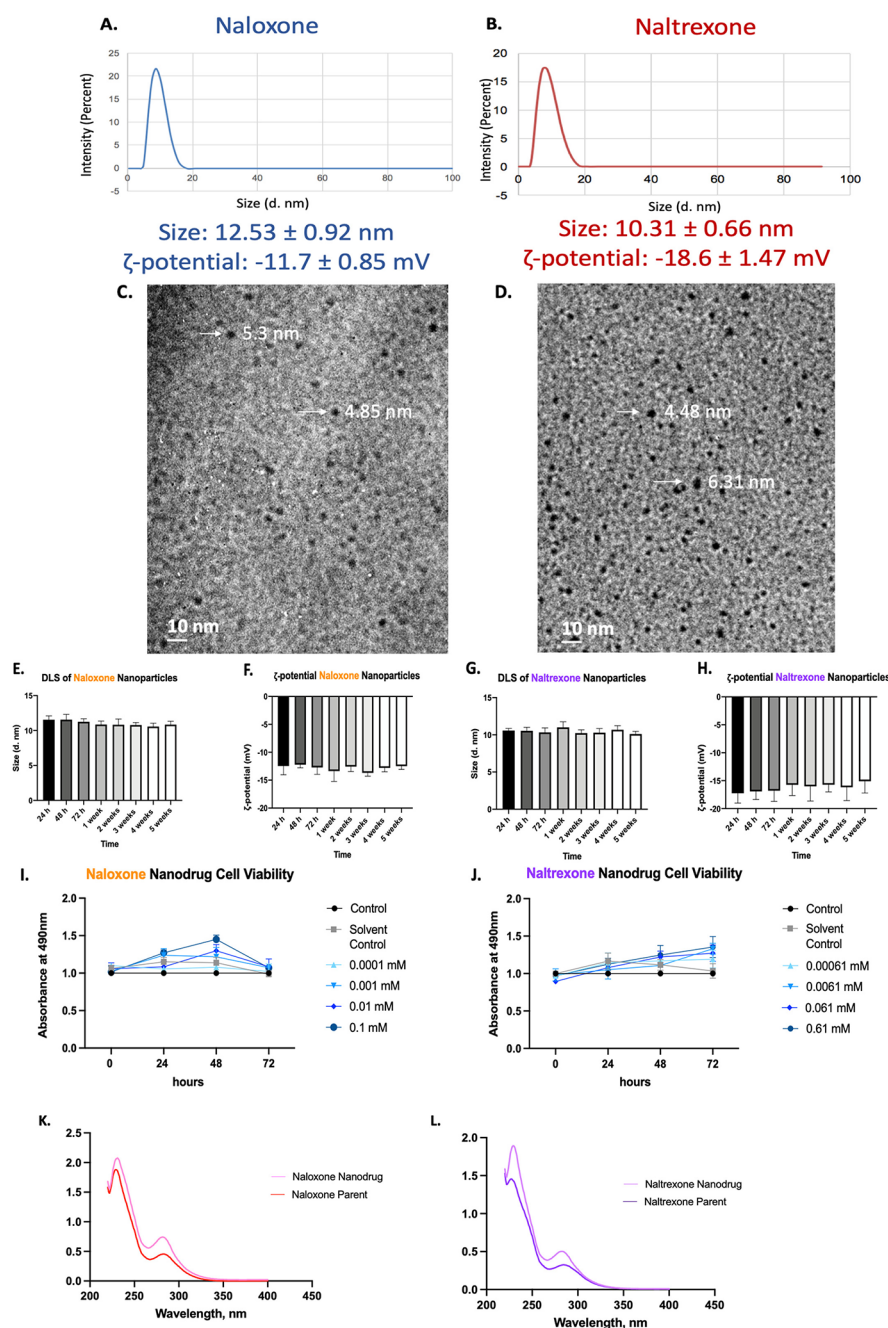


Figure 1. Nanoparticle characterization and assessment of cytotoxicity of nanodrugs. (A,B) Representative DLS⁴² graph of the hydrodynamic size of nanoparticles indicating monodispersed and reproducible nanoparticles. (A) Size of naloxone nanoparticles: 12.53 ± 0.92 nm. (B) Size of naltrexone nanoparticles: 10.31 ± 0.66 nm. Representative TEM image confirming the size and shape of (C) naloxone nanoparticles and (D) naltrexone nanoparticles. (E) DLS and (F) ζ -potential of naloxone nanoparticles measured across 5 weeks. (G) DLS and (H) ζ -potential of the naltrexone nanoformulation measured across 5 weeks. (I,J) MTS proliferation assay to ensure nontoxicity of nanoparticle formulations at varying concentrations in PC-12 cells. UV spectra for (K) naloxone, naloxone nano and (L) naltrexone, naltrexone nano. Data in (A,B,E–J) are mean \pm SEM. One-way ANOVA was used to analyze the significance of difference, and $P < 0.05$ was considered significant. All experiments were repeated at least three times for stability studies, $n = 4$, and for cell viability studies, $n = 8$ per group.

artery for 60 min. Afterward, the suture was removed and blood flow restored. Brains were harvested at 24 h post-reperfusion, sliced with a 1 mm brain matrix (Braintree Scientific) and stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC, ThermoFisher) in PBS solution. The images were captured with a digital camera, and the infarct volume in each brain slice was measured using Image J Software (NIH) and summed to calculate infarct volumes for each animal.

Data and Statistical Analysis. Statistical analyses were performed with GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). Experimental treatments were compared by one-way ANOVA followed by Tukey's multiple comparisons or Welch *t*-test with a significant value of $p < 0.05$. Data are mean \pm SEM.

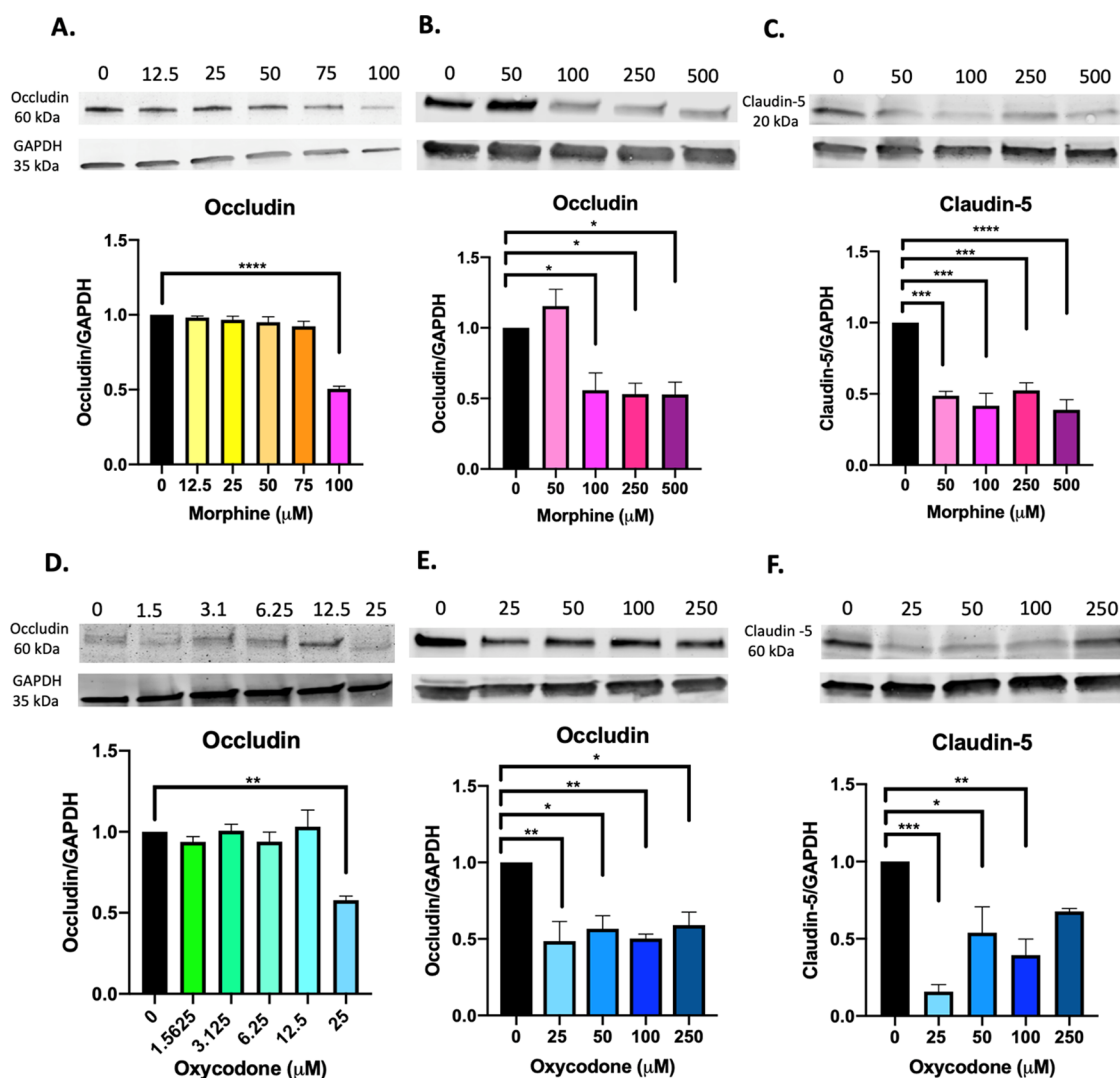


Figure 2. Morphine- and oxycodone-induced TJ protein dysregulation. (A,B) Expression of occludin and (C) claudin-5 protein in hCMEC/D3 cells after two treatments, 12 h apart with morphine (12.5, 25, 50, 75, 100, 250, 500 μM each), as analyzed by immunoblotting. (D,E) Occludin and (F) claudin-5 expression after two doses of oxycodone (1.5625, 3.125, 6.25, 12.5, 25, 50, 100, 250 μM each) 12 h apart in hCMEC/D3 cells. Data were normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) housekeeping protein expression and to control group of no opioid treatment (0 μM of the respective opioid concentration). Data in (A–F) are mean \pm SEM. One-way ANOVA was used to analyze the significance of difference as compared to the respective control. * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$), and **** ($P < 0.0001$) were considered significant. All experiments were repeated at least three times independently, $n = 6$ per group.

RESULTS

Characterization of Naloxone and Naltrexone Nanodrugs. Carrier-free naloxone and naltrexone nanodrugs were synthesized and characterized. DLS data indicate a uniform hydrodynamic size for both naloxone and naltrexone nanoparticles. Indeed, naloxone and naltrexone nanoparticles were $10.31 \text{ nm} \pm 0.66 \text{ nm}$ and $12.53 \text{ nm} \pm 0.92 \text{ nm}$ in size, respectively, and the narrow peaks in the DLS graph indicate homogeneity of size (Figure 1A,B). ζ -Potential was obtained to measure the electrical charge of the nanoparticles as a direct relation to their stability in the colloid suspension. ζ -Potential of $-11.7 \text{ mV} \pm 0.85 \text{ mV}$ for naloxone nanoparticles and $-18 \text{ mV} \pm 1.47 \text{ mV}$ for naltrexone nanoparticles was obtained, indicating suspension stability of the synthesized nanoformulations (Figure 1A,B). Representative TEM images revealed spherical shapes and sizes between ~ 3 and 5 nm for the generated naloxone and naltrexone nanoparticles (Figure 1C,D). The differences in nanoparticle sizes reported

between DLS and TEM are attributed to DLS measurements determining the true state of the nanoparticles in the solvent, referred to as the hydrodynamic size.^{50,51} Therefore, reported DLS nanoparticle size measurements are larger than those observed in TEM due to the inclusion of the solvent sphere around the nanoparticles. Nanoparticles were also evaluated for stability (Figure 1E–H). DLS of naloxone nanoparticles measured at multiple time points across 5 weeks indicated no changes in size over time (Figure 1E). ζ -Potential of the naloxone nanoformulation also measured at multiple time points across 5 weeks indicated no fluctuations in electrical charge of nanoparticles over time (Figure 1F). Similar stability results were obtained for naltrexone nanodrug (Figure 1G,H). Cell viability (MTS proliferation assay) was also conducted to evaluate the potential toxicity of generated nanoparticle formulations of naloxone and naltrexone in PC12 cultures. No differences in cellular viability were observed among treatments with naloxone or naltrexone nanodrug concen-

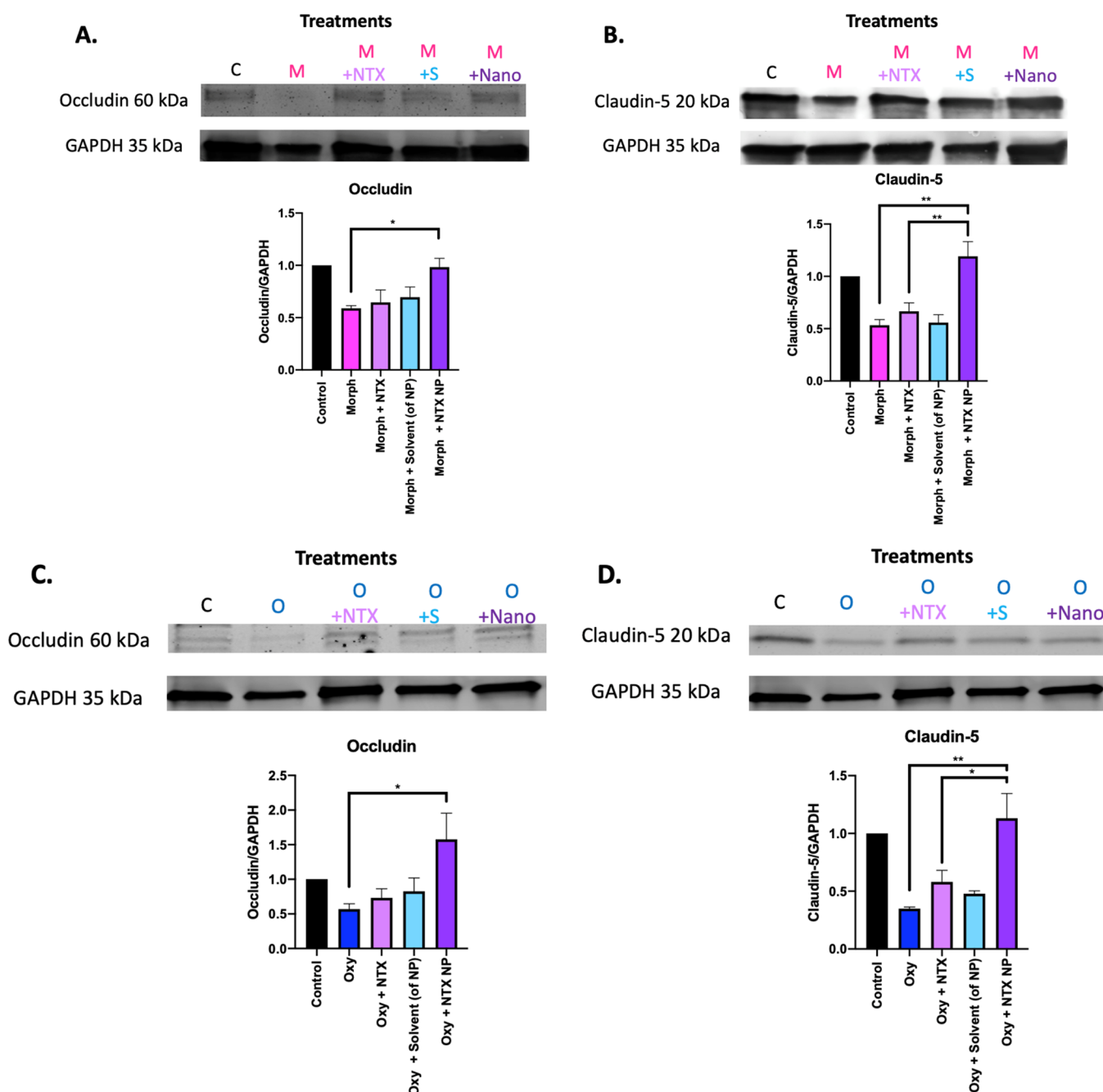


Figure 3. Naltrexone nanoparticles attenuate occludin and claudin-5 protein dysregulation. (A) Occludin expression in hCMEC/D3 cells exposed to morphine (100 μ M), naltrexone (0.125 mM), solvent control (S), and naltrexone nanoparticles (0.125 mM) as analyzed by immunoblotting. (B) Claudin-5 expression in the same samples as in (A). (C) Occludin expression in hCMEC/D3 cells exposed to oxycodone (25 μ M), naltrexone nanoparticles, and respective controls at the concentrations used in (A). (D) Claudin-5 expression in the same samples as in (C). Data in (A–D) are mean \pm SEM. One-way ANOVA followed by Tukey's multiple comparisons was used to analyze the significance of difference as compared to the respective controls. * ($P < 0.05$) and ** ($P < 0.01$) were considered significant. All experiments were repeated at least three times independently, $n = 6$ per group. Abbreviations: morphine (Morph, M), oxycodone (Oxy, O), naltrexone (NTX), nanoparticle (NP).

trations, suggesting no toxic effects of nanodrugs irrespective of concentration (Figure 1I,J). Furthermore, UV spectra were also comparable between the nanoparticle forms of the opioid antagonists and their respective parent forms (Figure 1K,L).

Opioid-Induced TJ Dysregulation. A range of morphine and oxycodone concentrations were evaluated to establish the LD₅₀ of the opioids for the disruption of TJ proteins occludin and claudin-5 (Figure 2). Concentrations tested were chosen based on the dosages used in patients with respect to the

estimated levels reaching the brain. Two treatments of 100 μ M morphine, administered 12 h apart, significantly altered occludin protein levels (Figure 2A,B) and claudin-5 protein levels in hCMECs (Figure 2C). Similarly, two treatments of 25 μ M oxycodone, administered 12 h apart, significantly reduced occludin and claudin-5 protein expression (Figure 2D–F).

Naltrexone and Naloxone Nanodrugs Attenuate Opioid-Induced Occludin and Claudin-5 Protein Dysregulation. Next, we proceeded to evaluate the hypothesis that

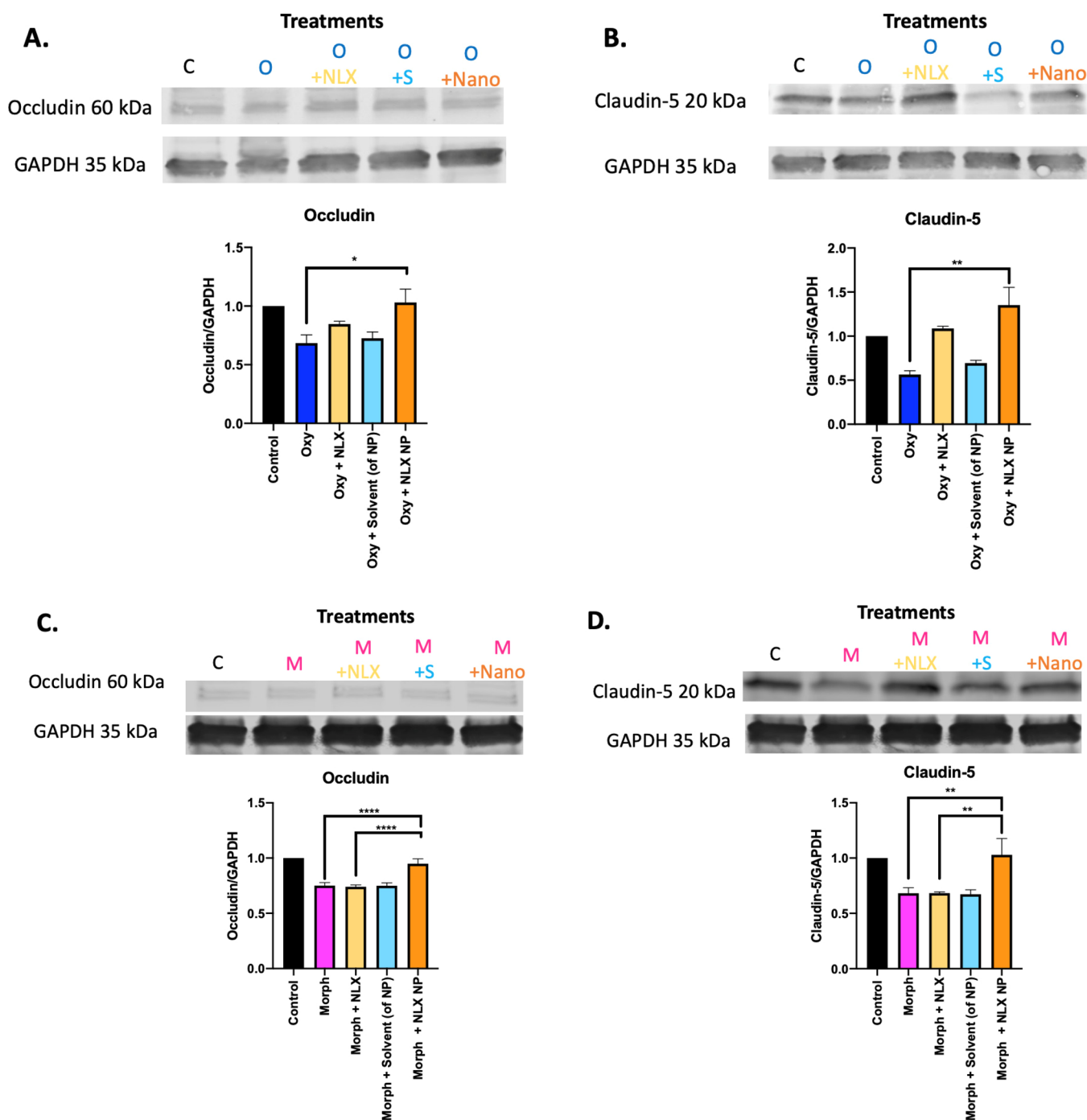


Figure 4. Naloxone nanoparticles attenuate occludin and claudin-5 protein dysregulation. (A) Occludin expression in hCMEC/D3 cells exposed to morphine (100 μ M), naloxone (0.001 mM), solvent control (S), and naloxone nanoparticles (0.001 mM) as analyzed by immunoblotting. (B) Claudin-5 expression in the same samples as in (A). (C) Occludin expression in hCMEC/D3 cells exposed to oxycodone (25 μ M), naloxone nanoparticles, and respective controls at the concentrations used in (A). (D) Claudin-5 expression in the same samples as in (C). Data in (A–D) are mean \pm SEM. One-way ANOVA followed by Tukey's multiple comparisons was used to analyze the significance of difference as compared to the respective controls. * ($P < 0.05$) and ** ($P < 0.01$) were considered significant. All experiments were repeated at least three times. Abbreviations: naloxone (NLX).

the generated opioid antagonist nanodrugs may attenuate opioid-induced TJ dysregulation. We tested both naloxone and naltrexone nanoformulations for protection against morphine- or oxycodone-induced alterations of occludin and claudin-5 expression (Figures 3 and 4). Naloxone and naltrexone in non-nanoparticle formation were also included to compare the therapeutic efficacy of the opioid antagonists both in nanoparticles and in native form. The solvent used during

the nanoparticle generation was used as an additional control to ensure that therapeutic effects were attributed to the opioid antagonists. Oxycodone-treated hCMEC/D3 cells that were also treated with naltrexone nanodrugs exhibited significant protection of occludin protein compared to oxycodone-treated hCMEC/D3 cells that were not treated with the nanodrugs (Figure 3A). Similarly, naltrexone nanodrug also provided protection against oxycodone-induced alterations of claudin-5

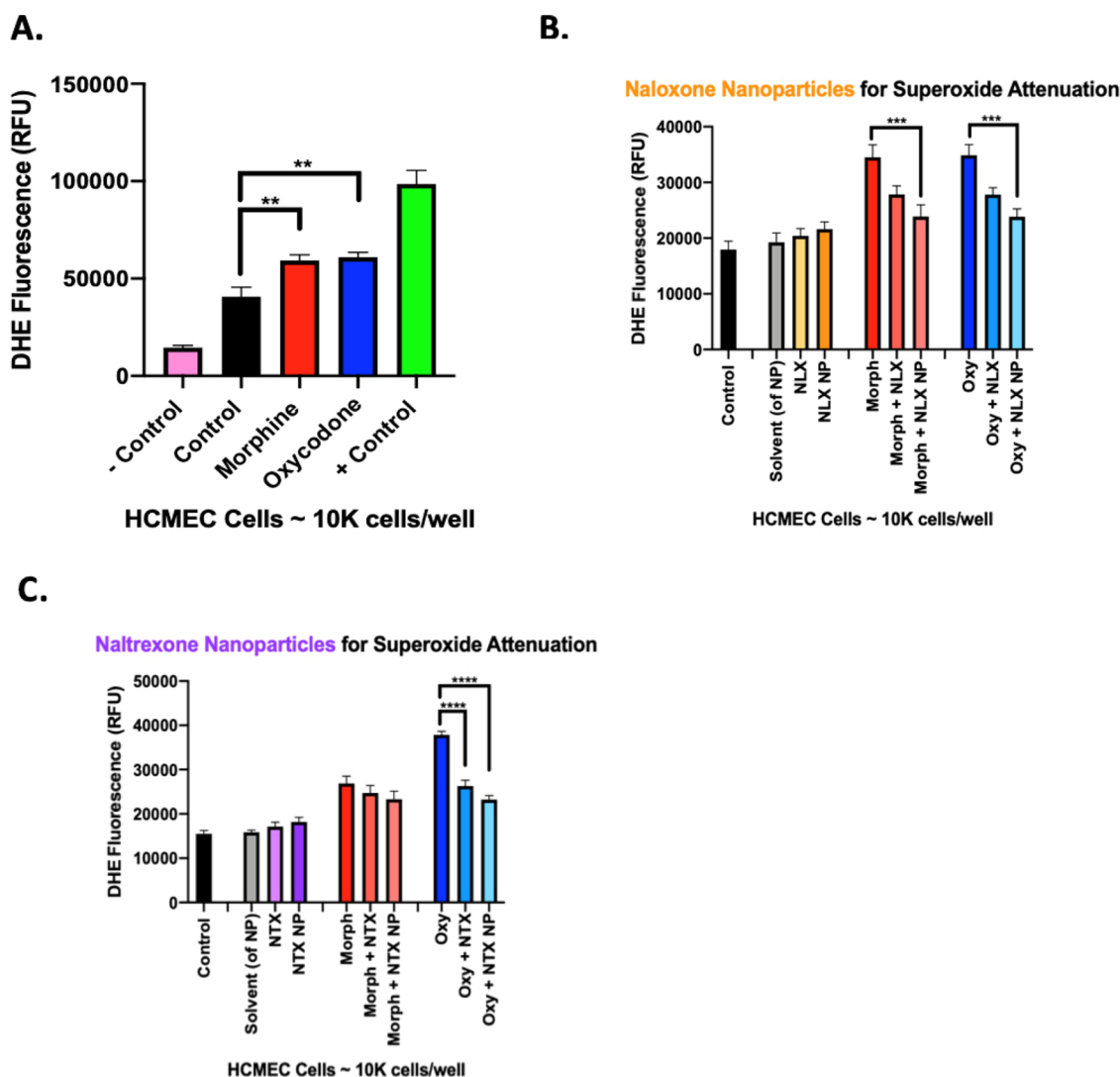


Figure 5. Opioid induced superoxide generation and subsequent attenuation by opioid antagonist nanoparticles. (A) Superoxide production was directly measured in cultured hCMEC/D3 cells exposed to morphine ($100 \mu\text{M}$) and oxycodone ($25 \mu\text{M}$) using a dihydroethidium (DHE) fluorescent probe. Attenuation of morphine- and oxycodone-induced superoxide generation by (B) naloxone nanoparticles (0.001 mM) and (C) naltrexone nanoparticles (0.125 mM). *N*-Acetyl cysteine was included as a negative control and antimycin A was included as a positive control for superoxide generation. Data are expressed as relative fluorescence units (RFUs) in mean \pm SEM. One-way ANOVA followed by Tukey's multiple comparisons or Welch's *t*-test was used to analyze the significance of difference as compared to the respective controls. * ($P < 0.05$) and ** ($P < 0.01$) were considered significant. All experiments were repeated at least three times independently, $n = 24$ per group.

levels in hCMEC/D3 (Figure 3B). Furthermore, there was a significant protection of claudin-5 protein levels in oxycodone-induced claudin-5 dysregulated cells treated with the nano-naltrexone compared to the same concentration of naltrexone, demonstrating increased efficacy of naloxone as a nanoformulation (Figure 3B). A similar attenuation of morphine-induced TJ dysregulation was observed in cells treated with the naltrexone nanodrug but not with naltrexone in native form (Figure 3C,D).

Similar to naltrexone, naloxone nanoparticles were also evaluated for their mitigation of oxycodone- or morphine-induced occludin and claudin-5 protein dysregulation. Exposure to naloxone nanodrug successfully provided protection against both oxycodone- and morphine-induced disruption of occludin and claudin-5 (Figure 4A–D). While native (i.e., not in nanoparticle formulation) naloxone was also protective, its effects were less pronounced compared to that of naloxone nanoparticles at the same concentration, indicating

increased therapeutic efficacy of the generated opioid antagonist nanoformulations in comparison to the native form of the drug.

Naloxone and Naltrexone Nanodrugs Reduce Opioid-Induced Superoxide Production via the NADPH Oxidase and TLR4 Mechanisms. In order to better understand the underlying pharmacodynamics attributed to the successful mitigation of TJ disruption by the generated nanoformulations, we then evaluated naloxone and naltrexone nanodrugs for the reduction of opioid-induced superoxide production. Significant production of reactive oxygen species, namely, superoxide, was observed after treatment with morphine and oxycodone at concentrations used to induce TJ dysregulation (Figure 5A). Subsequently, naloxone and naltrexone nanodrugs were then assessed for the reduction of opioid-induced superoxide (Figure 5B,C). Treatment with naloxone nanodrugs was highly effective in reducing morphine- or oxycodone-induced superoxide pro-

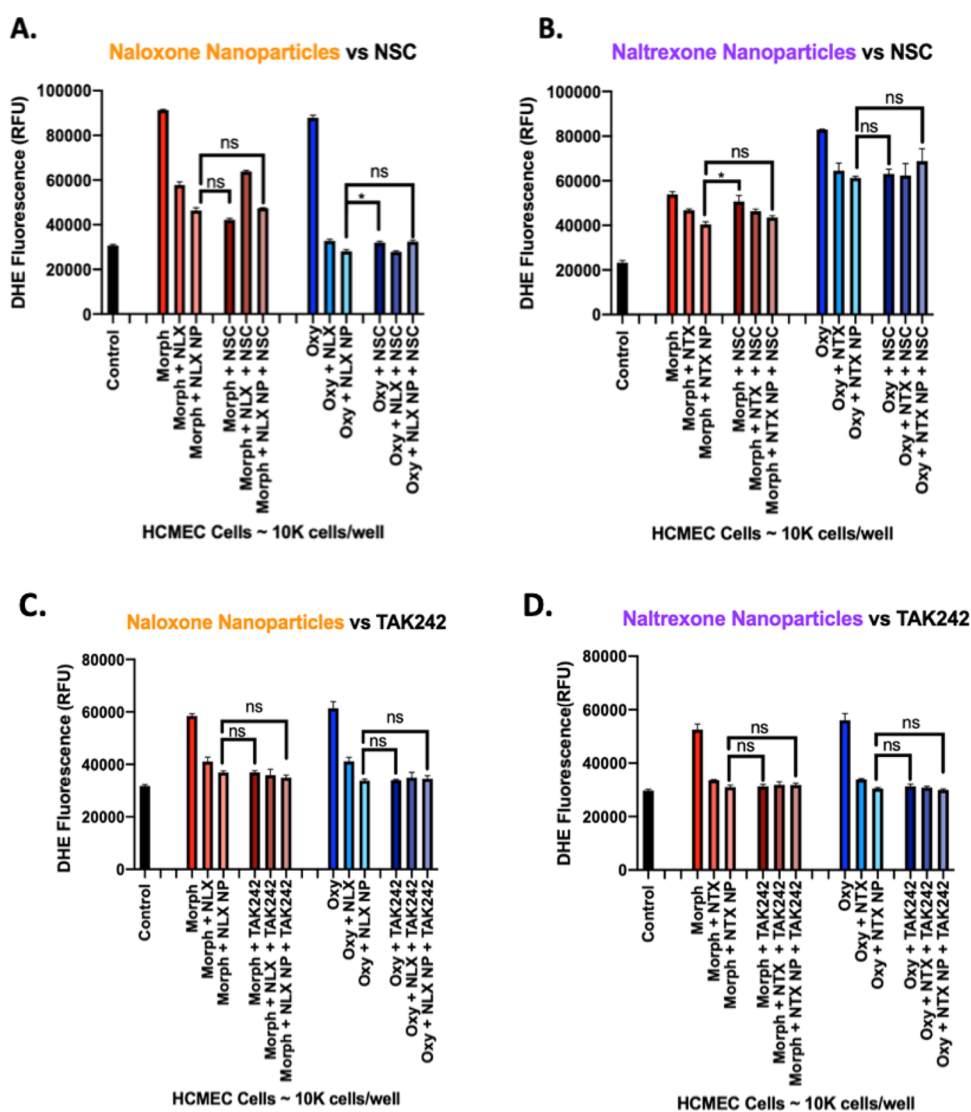


Figure 6. Naloxone and naltrexone nanoparticles attenuate superoxide generation comparable to the NADPH oxidase complex inhibitor (NSC) and TLR4 inhibitor (TAK242). Attenuation of morphine ($100 \mu\text{M}$)- or oxycodone ($25 \mu\text{M}$)-induced superoxide in hCMEC/D3 cells exposed to (A) 0.001 mM naloxone nanoparticles and/or NSC, (B) 0.125 mM naltrexone nanoparticles and/or NSC, (C) 0.001 mM naloxone nanoparticles and/or TAK242, and (D) 0.125 mM naltrexone nanoparticles and/or NSC. Data in (A–D) are mean \pm SEM. One-way ANOVA followed by Tukey's multiple comparisons or Welch's *t*-test was used to analyze the significance of difference as compared to the respective controls. * ($P < 0.05$) and ** ($P < 0.01$) were considered significant. All experiments were repeated at least three times independently, $n = 24$ per group. Abbreviations: NADPH oxidase complex inhibitor (NSC), TLR4 inhibitor (TAK242).

duction (Figure 5B). Similarly, cells treated with naltrexone nanoparticles exhibited a significant reduction of oxycodone-induced superoxide and showed a trend in attenuating morphine-induced superoxide production (Figure 5C).

The NADPH (dihyronicotinamide adenine dinucleotide phosphate) oxidase (NOX2) complex is an enzymatic complex involved in the induction of oxidative stress and is a plausible mechanism by which opioid antagonists may induce their respective neurorestorative and neuroprotective effects. Therefore, we compared superoxide attenuation by opioid antagonist nanodrugs to attenuation offered by NSC 23766 (NSC), an established pharmacological inhibitor of the NADPH oxidase complex. Reduction of morphine-induced superoxide production in hCMEC/D3 cells treated with the naloxone nanoformulation is comparable to the reduction offered from NSC (Figure 6A). Treatment with naloxone nanodrug offered greater protection from oxycodone-induced superoxide

production in comparison to treatment with NSC. Additionally, treatment with naloxone nanoparticles plus NSC was as protective against morphine- or oxycodone-induced superoxide production as exposure to naloxone nanoparticles alone (Figure 6A).

Naltrexone nanoparticles attenuated morphine-induced superoxide production to a greater extent in comparison to treatment with NSC (Figure 6B). In addition, they were equally protective in attenuation of oxycodone-induced superoxide as NSC. A combined treatment with naltrexone nanoformulations plus NSC had similar protective effects as NSC alone (Figure 6B). Overall, these results suggest that naloxone or naltrexone nanodrugs offer comparable or greater reduction of opioid-induced superoxide production than NSC.

The TLR4 (Toll-like receptor 4) signaling is another pathway that may serve as a source of opioid-induced superoxide production and contribute to neuroinflammation

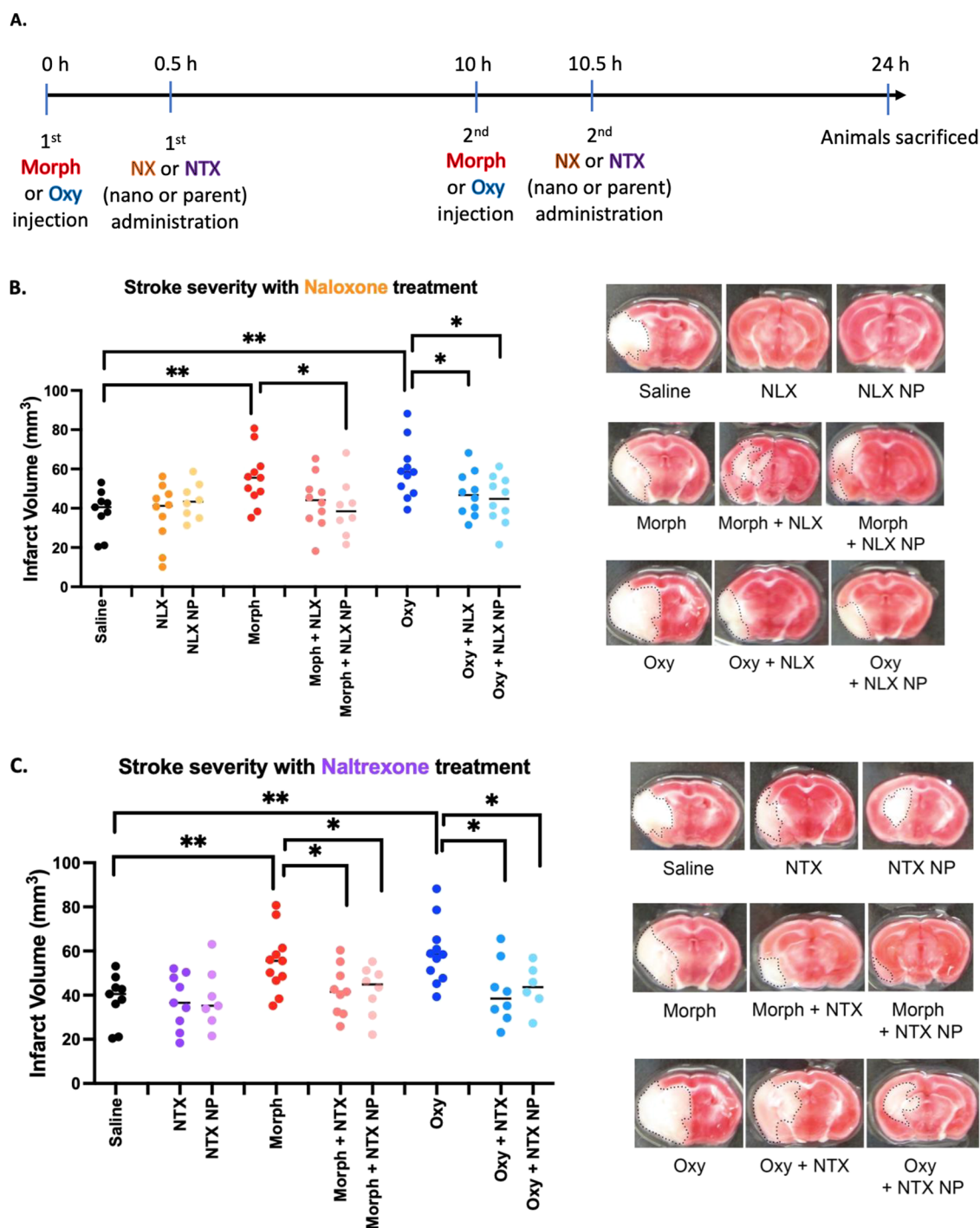


Figure 7. Opioid antagonist and opioid antagonist nanoparticles reduce stroke severity in mice. (A) Dose regimen methodology figure for MCAO animals. (B) Left panel, infarct volume; right panel, representative TTC images of mice exposed to morphine (40 mg/kg) or oxycodone (80 mg/kg) and treated with naloxone or naloxone nanoparticles (0.65 mg/kg). (C) Left panel, infarct volume; right panel, representative TTC stains of mice exposed to opioids and treated with naltrexone or naltrexone nanoparticles (2.2 mg/kg). One-way ANOVA followed by Tukey's multiple comparisons or Welch's *t*-test was used to analyze the significance of difference as compared to the respective controls. * ($P < 0.05$) and ** ($P < 0.01$) were considered significant. $n = 6$ – 10 animals per group.

and neurotoxicity in opioid abuse. Therefore, we evaluated the impact of the naloxone and naltrexone nanodrugs on this signaling pathway and compared their effects on attenuation of opioid-induced superoxide production to that mediated by a specific inhibitor of the TLR4 signaling pathway, TAK242 (Figure 6C,D). No differences in morphine- or oxycodone-induced superoxide diminution were found between naloxone nanoparticle treatment and exposure to the inhibitor of the

TLR4 pathway, TAK242. Additionally, treatment with both the naloxone nanoparticles and TAK242 did not offer additional protection against superoxide production compared to treatment with these drugs alone (Figure 6C). Similar patterns of changes were observed in experiments where naltrexone nanodrug attenuation of morphine- or oxycodone-induced superoxide production was compared to that of TAK242. Specifically, there were no differences between the

naltrexone nanodrug, TAK242, and/or using both agents in concert to protect against morphine- or oxycodone-stimulated superoxide production (Figure 6D). Taken together, these results signify the ability of the opioid antagonist nanodrugs to reduce superoxide comparably to an inhibitor of the TLR4 pathway, suggesting that the opioid antagonists in nanoformulations may induce their neuroprotective effects by modulation of the TLR4 signaling pathway.

Naloxone and Naltrexone Nanodrugs Reduce Stroke Severity in Opioid-Exposed Mice. Naloxone and naltrexone nanodrugs were finally evaluated in vivo for neuroprotection in mice exposed to opioids with an experimentally induced stroke. Briefly, mice received two injections of either morphine or oxycodone in 10 h intervals, followed by administration of naloxone or naltrexone in their native or nanodrug form 30 min after each injection with opioids (Figure 7A). An experimental stroke model was induced 24 h after the first injection with opioids by the MCAO for 60 min, followed by reperfusion. Brains were analyzed for stroke volume 24 h after the MCAO procedure (Figure 7A).

Morphine- or oxycodone-treated animals exhibited a significant increase in infarct volumes compared to saline-treated⁵² mice. Treatment with naloxone significantly reduced the infarct volume in the morphine-treated mice only when administered as nanoformulation (Figure 7B). Naloxone in its native form did not affect the morphine-induced potentiation of stroke volume, indicating lower therapeutic efficacy as compared to the nanoform. On the other hand, both naloxone and naloxone nanodrugs were equally protective in reducing the stroke volume in oxycodone-exposed mice (Figure 7B). Naltrexone and naltrexone nanoparticles were also tested for efficacy in the ischemic stroke model (Figure 7C). Naltrexone and naltrexone nanodrug were equally effective in protection against morphine- or oxycodone-induced stroke severity (Figure 7C). Both forms of naltrexone reduced the stroke volume to control levels; thus, no differences in therapeutic efficacy between naltrexone in its native form and nanodrug form were observed. Control animals that were not exposed to any opioid treatment but just received naloxone or naltrexone in their regular or nanoforms were also included to ensure nontoxicity of the opioid antagonists and opioid antagonist nanoformulations in vivo (Figure 7B,C).

DISCUSSION

With highly addictive physiological and psychological properties, opioids have set the precedent for the US opioid epidemic. Chronic prescription opioid use is linked to a higher risk for stroke, and currently, there are no FDA-approved pharmacological agents to attenuate the resulting pathological damage or reduce stroke severity. Pathologically, opioids disrupt the integrity of the BBB by downregulating fundamental TJ proteins and leading to neuroinflammation and neurodegeneration.^{22,25} To address the deficiency in stroke pharmacological options in the context of opioid abuse, we proposed a novel, yet unexplored application and repurposing of FDA-approved opioid antagonists, naloxone and naltrexone, as a prospective neuroprotective therapeutic strategy to minimize TJ dysfunction and reduce stroke severity.⁵³ Traditionally, these opioid antagonists are used to rescue and treat opioid abuse patients.⁵⁴ Naloxone is FDA-approved for the treatment of opioid overdose, and naltrexone is prescribed for the treatment of opioid addiction.^{55,56} Consequently, this research implements new strategies and advances in nano-

technology-based drug delivery methods, as well as uncharted drug repurposing of FDA-approved therapeutics to attenuate ischemic stroke severity by targeting TJ dysregulation incurred from chronic prescription opioids. We have developed carrier-free nanodrugs of naloxone and naltrexone that preserve the therapeutic properties of the original drugs. Use of nanotechnology is suggested to improve the delivery of drugs and enhance therapeutic potential while ultimately reducing the dose and frequency of dose required for therapeutic efficacy.³³ The flexibility in controlling shape and size during nanodrug preparation allows tailoring of characteristics for targeting delivery to the location of interest, like the TJ proteins of the BBB.^{33,34}

Herein, we have shown a successful and reproducible nontoxic, carrier-free formulation of naloxone and naltrexone nanoparticles. Pathologically, the generated opioid antagonist nanoparticles were demonstrated to significantly attenuate morphine- or oxycodone-induced occludin or claudin-5 TJ protein dysregulation. Furthermore, naloxone and naltrexone nanoparticles showed improved therapeutic efficacy through superior TJ protein attenuation in comparison to the non-nanoparticle form of the opioid antagonists. In vivo evaluation of opioid antagonists and opioid antagonist nanoparticles also supported our hypothesis of the neuroprotective abilities of naloxone and naltrexone in a stroke model. As opioid-treated animals that received the opioid antagonist or opioid antagonist nanoformulation exhibited a significantly reduced stroke volume, these preliminary results supported the therapeutic potential of our generated nanoformulations and their respective parent drugs.

While the mechanisms by which opioid antagonists naloxone and naltrexone induce their protective effects are yet to be fully understood, an inhibition of the NADPH oxidase and an impact on the TLR4 signaling have been suggested.⁷ Indeed, expression of the NADPH oxidase and TLR4 has been well characterized in brain endothelial cells.^{46,47} The NADPH oxidase enzymatic complex catalyzes superoxide production and is composed of a membrane-bound gp91phox subunit and p22phox, as well as three cytosolic proteins: p40phox, p47phox, and p67phox.⁵⁷ During an ischemic stroke, these cytosolic proteins are translocated across the plasma membrane to assemble an active NADPH oxidase enzyme complex with gp91phox and p22phox, in turn, increasing superoxide O_2^- production and oxidative stress.⁵⁸ Our results indicate that the generated nanoformulations of opioid antagonists effectively reduce morphine- or oxycodone-induced superoxide production. Importantly, protection against superoxide generation by the generated opioid antagonist nanodrugs is equal to that exerted by NSC, a specific pharmacological inhibitor of the NADPH oxidase complex. Subsequently, a combined treatment with opioid antagonist nanoformulation plus NSC did not offer any significant additional attenuation of superoxide. While naloxone and naltrexone in non-nanoformulation form also provided protection against morphine- or oxycodone-induced superoxide production, these effects were less pronounced as compared to nanodrugs. Taken together, these results indirectly suggest that the opioid antagonists or their respective nanoformulations may inhibit the enzymatic activity of the NADPH oxidase by binding to the gp91phox subunit and inducing a conformational change of the NADPH protein complex, affecting the binding affinity of the cytosolic subunits, p40phox, p47phox, and p67phox.^{7,59}

The TLR4 signaling pathway is activated as an innate immunity response to ischemic strokes, inducing microglial activation, cytokine production, and neuroinflammation.^{60,61} Few studies have shown that TLR4-deficient mice or mice treated with an anti-TLR4 antibody exhibit reduced infarct volumes, and therefore lesser stroke severity, compared to wild-type mice.^{62,63} This neuroprotection offered by ablating or blocking TLR4 yields fewer recruited inflammatory and immune response cells to the site of injury, thus protecting the already injured site from further neurotoxic mediators such as inflammatory cytokine, tumor necrosis factor alpha (TNF- α).⁶⁴ To that extent, however, inhibition of TLR4 signaling using naloxone and naltrexone for neuroprotection remains an unexplored therapeutic avenue for stroke.⁶⁵ To that extent, our results demonstrate that the naloxone and naltrexone nanoformulations offer comparable reduction of morphine- or oxycodone-induced superoxide generation to a small-molecule TLR4 inhibitor, TAK242. Additionally, treatment with both the TAK242 and opioid antagonist or opioid antagonist nanoformulation did not offer additional superoxide reduction. These novel results support the potential of the opioid antagonists and pure-drug opioid antagonist nanoformulations as TLR4 antagonists by preventing opioid engagement with the membrane-bound TLR4, leading to decreased immune activation and decreased inflammatory cytokine production.^{66,67}

In summary, through this work, we demonstrated the formulation of carrier-free, nontoxic, pure nanodrugs of naloxone and naltrexone that demonstrated efficacy in the reduction of opioid-induced TJ downregulation. Furthermore, the results of the present study indicate opioid antagonists' naloxone and naltrexone, and their respective nanoformulations, as promising therapeutic agents for protection against oxidative stress. Preliminary *in vivo* data in an animal model demonstrated reduction of stroke severity by the formulated nanoparticles in an opioid-induced ischemic stroke model. While the *in vivo* data primarily serves as a proof of concept demonstrating successful reduction of stroke, further studies evaluating the effectiveness of these nanoparticles as potential ischemic stroke therapeutics will be necessary, including larger-scale animal studies with an extensive histology, pharmacokinetic profiles, dose optimization, and biodistribution analyses. This study demonstrated premise for naloxone and naltrexone as therapeutics not only used for the treatment of opioid abuse and/or overdose but also for protection against opioid-associated cerebral vascular disorders, such as stroke. Ultimately, this work addresses the deficiency in effective pharmacological options for opioid-induced ischemic stroke and supports the novel application and repurposing of FDA-approved opioid antagonists, naloxone and naltrexone, in nanoformulations, as prospective stroke therapeutics.

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Notes

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