

**THERAPEUTIC EFFECTS OF MESENCHYMAL STEM CELL-DERIVED
SECRETOME ADMINISTRATION ON MORPHINE-INDUCED CONSUMPTION,
RELAPSE, AND WITHDRAWAL IN RATS:
ROLE OF BRAIN OXIDATIVE STRESS AND NEUROINFLAMMATION.**

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Thesis presented to the School of Medicine Clínica Alemana-Universidad del Desarrollo in
order to apply for doctoral degree (PhD) in Sciences and Innovation in Medicine

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August 2023
SANTIAGO

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This dissertation is dedicated with gratitude and love.
To Natalia, my constant support, you are my beacon in the depths of this academic journey.
To the Rossel Varas family, your enduring support has been invaluable.
To my parents, María and Sergio, and my siblings, your unwavering faith has been my
foundation. To all my friends, your presence and encouragement have been my strength.
This achievement is not mine alone but shared with all of you, for without you, this journey
wouldn't have been possible

ACKNOWLEDGMENT

First and foremost, my deepest gratitude goes to Natalia, my unwavering pillar of strength, your unwavering support has been my guiding light throughout this academic journey. Your strength in overcoming your own personal challenges, even at times when I was consumed by the demands of my doctoral work, has been a testament to your resilience and love. Your journey, intertwined with mine, has been the foundation upon which this achievement stands.

I owe an immeasurable debt of gratitude to the Rossel Varas family. María Varas, a mother-in-law like no other, your unconditional love and support have been a constant source of strength. Enrique Rossel, though reserved, every piece of advice you've shared has been a pearl of wisdom. The loss of Mario Varas, the "Pater family", was a bitter pill to swallow. From him, I learned the values of honesty, kindness, and respect for every living being. His wife, Yolanda Ramirez, with the wisdom and grace reminiscent of a Himalayan monk, welcomed me with open arms during my undergraduate years as a mom. Sandra Varas is the aunt everyone wishes they had - a confidante, a partner-in-crime, direct yet loving, and an absolute master of life's lessons.

To my parents, María and Sergio, whose love and teachings laid the foundation for this journey. The loss of my father during my doctoral studies was a profound blow. Yet, in the aftermath of his passing, I felt his guiding hand in every experiment, and every challenge, ensuring I stayed the course. His spirit has been my silent companion, guiding me through the highs and lows of this journey. To my siblings, your constant encouragement and belief have been my guiding stars.

I extend my heartfelt thanks to all the educators and mentors who have shaped my academic journey. Special acknowledgement goes to Fernando Ezquer for his FONDECYT grant and invaluable mentorship, to Pablo Berrios and Lucia Hipolito for his guidance and support.

To my doctoral colleagues, Isabel Rada, Orlando Ramirez, and Carla Araya, I am eternally grateful for the shared moments, challenges, and camaraderie. Our collective journey has been enriched by each other's presence, and I wouldn't trade our time together for anything.

To my lab mates, whose shared knowledge and advice have been invaluable, I extend my gratitude to Lorena Lobos, David Contador, Jessica Martínez, Verónica Silva, and Agustín Richards "el pana lab", your contributions to my journey have been endless.

To every individual who has been a part of this journey, from those who provided feedback to those who simply offered a word of encouragement, thank you. This achievement is a testament to the collective effort, belief, and support of each one of you. The strength and faith bestowed upon me by all of you have been my spiritual anchor, giving me the motivation and resilience to rise above even the most challenging adversities.

In essence, this dissertation is not just a reflection of my work, but a tapestry woven with the threads of love, support, and belief of all those who have been a part of my journey. It is a shared achievement, a collective triumph, and a testament to the power of collaboration, belief, and perseverance.

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LIST OF ABBREVIATURES

MSC	Mesenchymal Stem Cell
MOR	μ -opioid receptor
DA	Dopamine
NAc	Nucleus accumbens
GLT-1	Glutamate transporter 1 / Excitatory amino acid transporter 2 (EAAT2)
ROS	Reactive oxygen species
CPP	Conditioned place preference
hMSC	Human Mesenchymal Stem Cell
GSSG/GSH	Ratio of oxidized glutathione to reduced glutathione
xCT-system	Antiporter system xc ⁻
mRNA	Messenger ribonucleic acid
RGS17	Regulator of G-protein signaling 17
UchB	Ethanol drinker Universidad de Chile rat B
CDC	Centers for Disease Control and Prevention
FONDECYT	Fondo Nacional de Desarrollo Científico y Tecnológico (Chilean National Fund for Scientific and Technological Development)
ANID	Agencia Nacional de Investigación y Desarrollo (National Agency for Research and Development, Chile)

1. INTRODUCTION AND DISSERTATION CONTEXT

The incidence of opioid addiction in Chile and the world has become a global crisis of monumental proportions, encompassing a vast and intricate landscape that engulfs our society. The consumption of opioids used medicinally for their analgesic and sedative properties in the management of clinical pain, has increased by 345% in the U.S. between 2001 and 2016 [1, 2]. However, the pursuit of the hedonistic effects of "well-being" and "euphoria," coupled with the non-therapeutic use of these substances, has led to a sharp rise in opioid abuse [3, 4]. In 2022 the CDC's National Center for Health Statistics reported an estimated 107,622 opioid overdose deaths in the United States during 2021, an increase of nearly 15% from the 93,655 deaths estimated in 2020, with opioids responsible for almost 70% of all drug overdose deaths [5, 6].

In South America, Chile's prevalence of opioid consumption, particularly among teenagers [7, 8], and the duplication of their consumption from 2015 to 2019 [9], make the situation especially disheartening. Relapse to opioid use is compounded by the pathological association of environmental perceptions with the drug's rewarding effects, and by the severity of the withdrawal syndrome [10, 11]. This multifaceted challenge has become a daunting problem for addiction science, with a dearth of enduring and efficacious interventions.

In the vast landscape of opioid addiction, a challenge of global magnitude, my doctoral research sought to understand the nuanced mechanisms of morphine consumption, withdrawal syndrome, and relapse. Once opioids are administered, they bind and activate endogenous opioid receptors in the brain, such as the μ -opioid receptor (MOR) [12], which has the cascading effect of promoting the release of dopamine (DA) in key brain areas associated with rewarding stimuli and the learning of environmental perceptions linked with drug use. This complex process lays the groundwork for the hedonistic effects and reinforcement properties that contribute to addiction [13, 14].

Beyond the initial receptor reinforcement, continuous administration of opioids results in a pathological pairing of environmental and subjective stimuli conditioned with opioid use. The omnipresence of these signals during abstinence periods can trigger a new round of drug administration, driving relapse and perpetuating opioid intake, a cycle that can persist for years [15]. The addictive nature of opioids, coupled with the severe physical dependence they engender—evidenced by the swift onset of a potent withdrawal syndrome when consumption ceases—make these drugs particularly insidious [11].

Chronic use of opioids results in oxidative stress and neuroinflammation in the brain, which further exacerbates addictive behaviors [16]. The potential causal role of oxidative stress and neuroinflammation in triggering withdrawal syndrome and prompting consumption with subsequent relapse is an area of study that has received significant attention [17-20]. Research has revealed that sustained abuse of drugs [21], opioids included, can induce synaptic alterations [22-24]. Central to these alterations are glial cells - microglia and astrocytes - that envelop the synapses and participate actively in their modulation [25, 26].

This interaction can lead to a deficit in the control of drug cravings, creating an environment conducive for dependency and subsequent relapse.

Further investigations have identified the vital role of glutamatergic balance in the nucleus accumbens (NAc) in driving cravings triggered by environmental cues [27-29]. Neuronal afferents from the prefrontal cortex and the hippocampus influence this homeostatic balance [30-32]. For drug-dependent individuals, there is a notable deficiency in the reuptake of the neurotransmitter glutamate-mediated by astrocytes, thereby inducing a hyperglutamatergic state in the NAc [33-35].

Within this context, the astrocyte glutamate transporter GLT-1, responsible for approximately 90% of glutamate uptake in the brain, becomes critically involved [33, 36]. Reactive oxygen species (ROS) can directly inhibit GLT-1 through the lipoperoxidation product 4-hydroxynonenal, which forms adducts with this transporter [37, 38]. Notably, oxidative stress and neuroinflammation are linked to a reduction in GLT-1 activity and levels [39-41]. The resultant decrease in GLT-1 in the NAc and the corresponding rise in extracellular glutamate stimulate the excitatory neurotransmission of the brain's reward system, leading to heightened cravings and potentially initiating relapse to opioid use [42]. However, promising observations from morphine-dependent animal models suggest that the administration of β -lactams (inducers of the expression of the glutamate transporter GLT-1), such as ceftriaxone, can reverse the withdrawal syndrome induced by naloxone [43]. This demonstrates the potential to inhibit the reinstallation of morphine in conditioned place preference (CPP) through stimulation of GLT-1 expression [44]. **Therefore, the reversal of glutamatergic dysregulation—induced by oxidative stress and neuroinflammation—presents an exciting therapeutic opportunity to reduce chronic morphine consumption, prevent withdrawal syndrome, and subsequently inhibit morphine relapse.**

In essence, therapies targeting the increase in oxidative stress and neuroinflammation caused by opioid abuse could present a groundbreaking strategy to decrease chronic opioid use, alleviate withdrawal syndrome, and preventing relapse [45, 46]. However, due to the novelty of these mechanisms and the challenge posed by the blood-brain barrier, there are no currently available treatments focused specifically on reducing oxidative stress and neuroinflammation induced by chronic opioid consumption [47-49].

Unfortunately, current therapeutic strategies, primarily based on forced abstinence, prove largely ineffective, with a staggering relapse rate of over 90% after the first month of abstinence [50]. Presently, substitution therapy remains the mainstay of opioid dependence treatment. Here, the consumption of high-potency opioids such as morphine are substituted by less potent opioids like methadone or buprenorphine, which are considered safer due to their longer half-lives. This therapy aims to reduce the opioid dose gradually. Nevertheless, it carries substantial side effects such as respiratory depressions [51] and show highly variable distribution among individuals, complicating personalized administration [52]. Additionally, these replacement therapies have been found to increase oxidative stress in peripheral blood [53], a process linked with the development of drug dependence [54]. Considering these findings, my research sought to identify more effective interventions for opioid addiction. To curb relapse and halt the progression of opioid dependence, strategies that focus on reducing chronic consumption, managing cravings, and preventing withdrawal

syndrome are crucial. The challenges inherent to this endeavor reflect the intricate interplay of mechanisms that underlie opioid addiction, a dynamic area I explored in my research to uncover novel insights and therapeutic opportunities [55, 56]

An emerging approach for the treatment of complex neurological diseases is mesenchymal stem cell (MSC)-based therapy. MSCs stand out for their antioxidant and anti-inflammatory properties [57], which can be intensified by subjecting them to preconditioning stimuli *in vitro* [58]. The molecules secreted by these cells (secretome) can be used as a powerful biopharmaceutical, increasing the biosafety profile of the treatment compared to the administration of living cells. It has recently been shown that intranasal administration of secretome derived from preconditioned human MSCs (hMSCs) in an animal model of alcohol addiction reduced chronic consumption and prevented relapse to consumption after a period of abstinence [59]. These therapeutic effects were associated with a reduction in brain oxidative stress and neuroinflammation in animals that consumed alcohol [59].

Stepping into this complex tableau, my doctoral research delved into the delicate underpinnings of opioid consumption, relapse, and withdrawal, aiming to unravel novel insights and dissect the intricate processes that underlie the crisis. These findings hold promise for transforming the landscape of opioid addiction treatment and opening new avenues for further exploration in addiction science

Based on these antecedents, this thesis presents the results obtained after the simultaneous intranasal and intravenous administration of the antioxidant and anti-inflammatory secretome generated from preconditioned hMSC on the precipitated (naloxone-induced) and spontaneous (oral model) withdrawal syndrome, the reduction in its chronic consumption, and the relapse to voluntary oral consumption (oral model) in rats exposed to continuous doses of opioids. Additionally, the association and role of oxidative stress and neuroinflammation in the different stages of morphine addiction were evaluated.

2. HYPOTHESIS

The simultaneous administration of hMSC-derived secretome reduces chronic consumption, relapse, and withdrawal symptoms in rats. These effects are associated with a reduction in opioid-induced brain oxidative stress, neuroinflammation and the normalization of glutamatergic homeostasis.

3. SPECIFIC OBJECTIVES

1. Determine whether the administration of the secretome derived from preconditioned hMSCs reduces chronic consumption, alleviates withdrawal symptoms, and inhibits relapse to consumption in rats exposed to morphine, while simultaneously normalizing glutamate homeostasis (evidenced by extracellular glutamate levels, GLT-1, and xCT-system).
2. Evaluate whether the administration of the secretome derived from preconditioned hMSCs diminishes brain oxidative stress (measured by the GSSG/GSH ratio and lipid peroxidation) in rats with chronic consumption, withdrawal symptoms, and during relapse to consumption triggered by morphine administration.
3. Investigate whether the administration of the secretome derived from preconditioned hMSCs reduces neuroinflammation (indicated by astrocytic and microglial density) in rats with chronic consumption, withdrawal symptoms, and during relapse to consumption induced by morphine administration.

4. Chapter 1

A Novel Morphine Drinking Model of Opioid Dependence in Rats

Access: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8999131/>



Article

A Novel Morphine Drinking Model of Opioid Dependence in Rats

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Citation: Berrios-Cárcamo, P.; Quezada, M.; Santapau, D.; Morales, P.; Olivares, B.; Ponce, C.; Ávila, A.; De Gregorio, C.; Ezquer, M.; Quintanilla, M.E.; et al. A Novel Morphine Drinking Model of Opioid Dependence in Rats. *Int. J. Mol. Sci.* **2022**, *23*, 3874. <https://doi.org/10.3390/ijms23073874>

Academic Editor: Zachary A. Rodd

Received: 22 February 2022

Accepted: 28 March 2022

Published: 31 March 2022

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Abstract: An animal model of voluntary oral morphine consumption would allow for a pre-clinical evaluation of new treatments aimed at reducing opioid intake in humans. However, the main limitation of oral morphine consumption in rodents is its bitter taste, which is strongly aversive. Taste aversion is often overcome by the use of adulterants, such as sweeteners, to conceal morphine taste or bitterants in the alternative bottle to equalize aversion. However, the adulterants' presence is the cause for consumption choice and, upon removal, the preference for morphine is not preserved. Thus, current animal models are not suitable to study treatments aimed at reducing consumption elicited by morphine itself. Since taste preference is a learned behavior, just-weaned rats were trained to accept a bitter taste, adding the bitterant quinine to their drinking water for one week. The latter was followed by allowing the choice of quinine or morphine (0.15 mg/mL) solutions for two weeks. Then, quinine was removed, and the preference for morphine against water was evaluated. Using this paradigm, we show that rats highly preferred the consumption of morphine over water, reaching a voluntary morphine intake of 15 mg/kg/day. Morphine consumption led to significant analgesia and hyperlocomotion, and to a marked deprivation syndrome following the administration of the opioid antagonist naloxone. Voluntary morphine consumption was also shown to generate brain oxidative stress and neuroinflammation, signs associated with opioid dependence development. We present a robust two-bottle choice animal model of oral morphine self-administration for the evaluation of therapeutic interventions for the treatment of morphine dependence.

Keywords: morphine; opioids; addiction; oral intake; animal model; dependence; quinine

1. Introduction

Opioids are widely used to treat acute and chronic pain due to their potent analgesic and sedative properties. However, since opioids also generate the compulsion to take the drug, it has led to widespread non-medical opioid use [1].

Opioid drugs are among the most addictive substances [1]. Repeated daily use leads to physical dependence and rapid tolerance to their effects [1]. Thus, opioid dependence has become an epidemic concern, with over 16 million opioid-dependent individuals

worldwide and over 130,000 deaths reported annually [2], representing 76% of deaths in which a drug-use disorder was implicated [3,4].

It is consistently reported that the oral route is the preferred route of administration for prescription opioids. Several studies show that over 90% of abusers of prescription opioids, including oxycodone, hydrocodone, and morphine, report oral ingestion for non-medical purposes [5,6]. Thus, the generation of animal models of voluntary opioid oral intake has become a necessary pre-clinical tool to evaluate new pharmacological strategies for the treatment of opioid dependence.

Studies evaluating the voluntary oral self-administration of drugs of abuse in animal models often utilize procedures in which the preference for drug-containing solutions is assessed against either water or palatable non-drug solutions. Among the different paradigms commonly used, the two-bottle free-choice exposure is widely used to evaluate drug avidity, being a non-invasive procedure in which animals have the option of voluntarily drinking the desired amount of drug. This paradigm has demonstrated relevant translational value for several drugs of abuse [7,8]. However, the main limitation of oral morphine intake is its bitter taste. Thus, when the morphine bottle is paired with a bottle containing non-adulterated tap water, animals prefer the non-morphine solution [9]. To overcome this limitation, researchers reported the addition of taste adulterants such as sweeteners, e.g., saccharin, added to the morphine solution or bitterants, e.g., quinine, in the alternative bottle to increase morphine consumption and to demonstrate morphine dependence [9,10]. However, the neurobiology by which quinine and saccharin influence the ensuing preference for morphine in rodent models remains poorly understood.

Ferraro et al. reported that the addition of 0.2% saccharin to the morphine solution could mask the bitter taste of morphine, increasing morphine preference compared to the control bottle [11]. However, morphine and saccharin have known pharmacological interactions since saccharin potentiates morphine-induced antinociception in mice [12]. Furthermore, saccharin consumption is also known to promote dopamine release in the nucleus accumbens, activating the mesolimbic reward system [13]. Even after several days of high intake of morphine plus saccharin, the elimination of saccharin from the bottles markedly reduces morphine consumption [11], thus limiting the translational value of this model. Similarly, rats given morphine adulterated with sugar in their drinking water for 21 days do not develop a preference for the morphine-sugar bottle when evaluated against a bottle with only sugar dissolved in water [14]. Thus, the taste-altering confounding factor must be eliminated before animals that consume morphine could be reliably used for pre-clinical testing of treatments aimed at reducing consumption elicited by morphine itself.

On the other hand, when quinine is included in the alternative bottle, a preference for the morphine solution is observed, suggesting that quinine is effective in driving morphine consumption and generating dependence. However, several reports demonstrated that, in this paradigm, high morphine preference is not related to the rewarding properties of the drug, but its preference is influenced by the degree of quinine aversion [15,16]. Thus, it was postulated that the aversion to the bitter taste of quinine is what leads to the maintenance of relatively high levels of fluid consumption from the morphine bottle [15]. This interpretation is supported by the significant reduction in morphine intake observed when quinine is eliminated from the alternative bottle, despite weeks of morphine consumption [11,15]. Thus, new paradigms to induce a preference for morphine-containing solutions without the influence of taste adulterant confounders are highly needed.

Rodents, similar to humans, are born with only a few innate flavor preferences and aversions, and they exhibit marked plasticity throughout the individual's lifespan, being highly sensitive to modification by experience [17]. In rodents, flavor preference learning usually occurs at the time of weaning and could influence food selection after weaning [17]. Thus, repeated exposure to an aversive flavor early in life could improve flavor acceptance in adult life due to familiarity, reducing inherent neophobia [18]. The bitter taste is one of these aversive flavors. Interestingly, exposure of young rats to the bitterant quinine as their only source of fluid immediately after weaning significantly increases quinine preference

when evaluated in a two-bottle choice against water [19]. Those results suggest that quinine consumption after weaning makes quinine, and possibly other bitterants, more palatable to weanling rats.

In this study, we show a novel morphine drinking paradigm in which, immediately after weaning, rats are exposed for seven days to a quinine solution as their only fluid source, to make animals get used to a bitter taste. After that period, animals are exposed to a two-bottle choice of quinine or morphine solutions for two weeks. Finally, the quinine bottle is replaced by a water bottle, and animals now have the choice of drinking morphine or water. After four weeks of voluntary morphine intake, we evaluated classical markers of the pharmacological action of morphine, including thermal and mechanical analgesia and hyperlocomotion, as well as markers of morphine dependence including the appearance of somatic signs of morphine withdrawal triggered by the administration of naloxone, an opioid receptor antagonist. In additional studies, we assessed other molecular markers of opioid-induced dependence, including morphine-induced neuroinflammation and oxidative stress and the mRNA levels of μ -opioid receptor and accessory proteins.

2. Results

2.1. Morphine Oral Consumption

Animals were trained at a young age to accept the bitter taste of quinine ($n = 29$). Upon weaning, three-week-old rats were exposed to a quinine solution (0.15 mg/mL) as their only drinking fluid for seven days. Despite the high quinine concentration, which is shown to produce significant suppression of fluid and food intake in adult rats [19], the consumption of quinine solution following the first two days of access was not significantly different from that of the water-only exposed animals ($n = 8$) (Figure 1A), and their body weight gains were indistinguishably between both groups (Figure 1B).

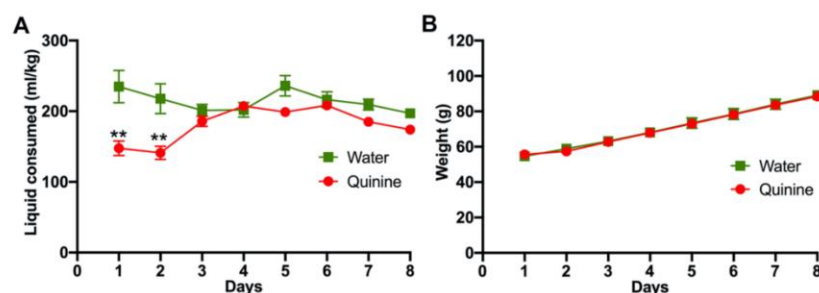


Figure 1. Young rats learn to drink a bitter-tasting quinine solution. Immediately after weaning, three-week-old female Wistar rats were exposed to water or 0.15 mg/mL quinine dissolved in water as the only fluid choice. (A) Water or quinine solution intake was evaluated daily and expressed as ml consumed/kg body weight. (B) Animal weights were determined daily. $n = 8$ in the water group and $n = 29$ in the quinine group. Data are expressed as mean $\pm$ SEM. ** $p < 0.01$, Two-way ANOVA.

Following seven days of quinine exposure, a morphine-solution (0.15 mg/mL) containing bottle was added in addition to the quinine-containing bottle (a water-only bottle was not available). A marked preference for the novel morphine solution was quickly observed, and the animals reached a ~90% preference for the morphine solution over the quinine solution after five days of the two-bottle choice for the bitter solutions (Figure 2A,B). Only four animals (14%) kept a significant quinine preference (below 50% preference for the morphine solution) and were removed from the study. While a modest downward intake of the morphine solution was seen thereafter, likely the result of the fast increases in body weight of recently weaned animals, the high preference for the morphine solution remained, which was consistent with a reduced total fluid consumption normalized by body weight (Figure 2C,D).

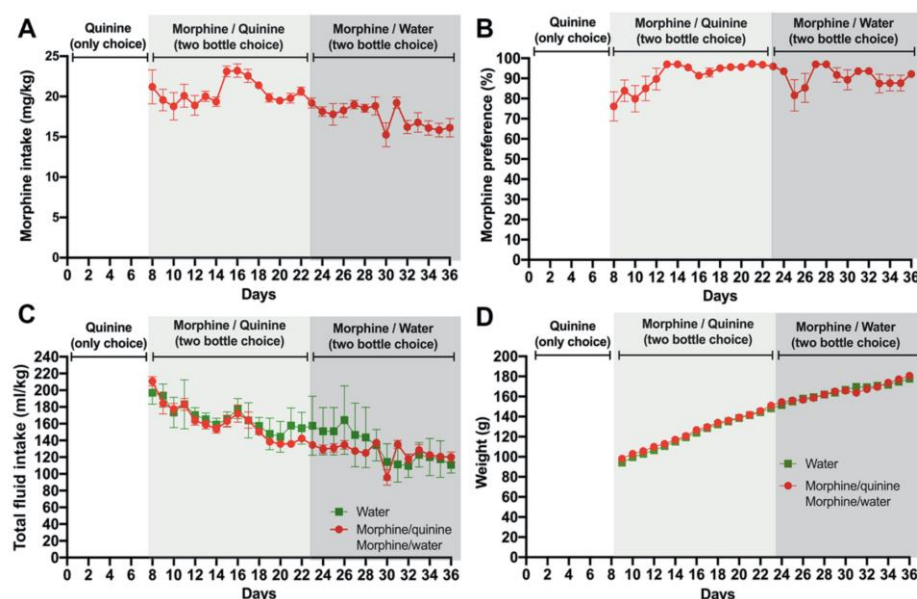


Figure 2. Voluntary morphine intake by Wistar rats. One week after starting quinine intake (Figure 1), animals were exposed to 0.15 mg/mL quinine and 0.15 mg/mL morphine for two weeks using the two-bottle choice paradigm. Thereafter, animals were exposed to 0.15 mg/mL morphine and water (two-bottle choice) for two additional weeks. (A) Voluntary morphine intake is expressed as mg morphine consumed per kg of body weight per day. (B) Morphine preference (%). (C) Total fluid intake expressed as total fluid consumed per kg of body weight/day. (D) Animal weights. Data are expressed as mean $\pm$ SEM. $n = 8$ in the water group, and $n = 18$ in the morphine group; 38% of the animals in the morphine group were excluded since they did not show >50% morphine preference against water.

After two weeks of free-choice between morphine and quinine solutions, the quinine bottle was replaced by one filled with only water to assess the morphine consumption without the confounding presence of quinine. Despite a small initial drop in preference for morphine, the preference for morphine stayed higher than 80% and, importantly, voluntary morphine intake stayed above 15 mg/kg/day for the remaining two weeks of observation (Figure 2A), reaching morphine plasma levels of 2.5 ± 0.6 mg/mL measured during the first hour of light, to expect the highest plasmatic morphine level, since rats mainly drink overnight. It is noted that seven animals changed their preference ratio from the morphine solution to water during the two weeks of observation and were removed from the study. Thus, the overall rejection corresponds to 11 animals of the original 29 (38%).

2.2. Orally Consumed Morphine Was Pharmacologically Relevant

To determine whether rats self-administering 15–18 mg morphine/kg/day experienced the pharmacological effects of morphine, we determined if the animals (i) showed morphine-induced analgesia, (ii) showed morphine-induced hyperlocomotion, and (iii) developed a physical dependence shown by somatic signs consistent with the opioid withdrawal syndrome, after the systemic administration of the opioid receptor antagonist naloxone [20,21].

(i) *Morphine-induced analgesia.* As a measure of the analgesic effect of morphine, rats that had voluntarily consumed morphine for four weeks were tested for their withdrawal latency following a thermal stimulus and for the withdrawal threshold to a mechanical stimulus. Morphine-consuming animals showed a significantly higher thermal withdrawal

latency (Figure 3A) and a significantly higher mechanical withdrawal threshold (Figure 3B) compared to water-drinking controls ($p < 0.0001$).

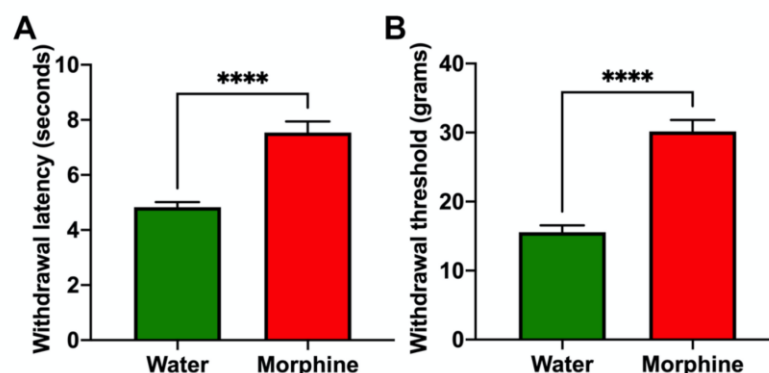


Figure 3. Voluntary morphine intake induces analgesia in Wistar rats. Hargreaves plantar test (A) and Von Frey test (B) showing the withdrawal latency (delayed response) to thermal stimulus and withdrawal threshold to mechanical stimulus, respectively, of rats that had voluntarily ingested a morphine solution for four weeks. Animals drinking only water were used as controls. Withdrawal latency and withdrawal threshold were measured during the first hour of light. Data are expressed as mean $\pm$ SEM. $n = 8$ in each experimental group. **** $p < 0.0001$, two-tailed Student t -test.

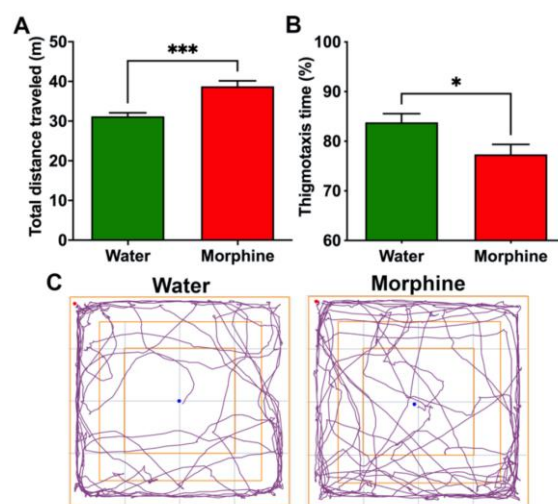


Figure 4. Voluntary morphine intake induces hyperlocomotor activity in Wistar rats. (A) Total distance traveled and (B) Thigmotaxis time (%) as a measure of time spent near the walls during the five minute session of the open field test of rats that had voluntarily consumed morphine for four weeks. Animals drinking only water were used as controls. (C) Trajectory plot of rats drinking morphine and water showing entries to the center of the open field. The open field test was conducted during the first hour of light. Data are expressed as means $\pm$ SEM. $n = 8$ in each experimental group. * $p < 0.05$; *** $p < 0.001$, two-tailed Student t -test.

(ii) *Morphine-induced hyperlocomotion.* Rats that had consumed morphine and were placed on an open field showed a higher distance traveled in five minutes ($p < 0.001$) compared to water-drinking rats (Figure 4A). The experimental rats also spent a lower percentage of the session close to the compartment walls, decreasing thigmotaxis compared

to control rats ($p < 0.05$) (Figure 4B,C), a measurement of anxiety reduced by morphine treatment [22].

(iii) *Morphine withdrawal syndrome.* Animals that had voluntarily consumed morphine also developed physical dependence. This was shown by the eliciting of withdrawal syndrome signs following the administration of the opioid antagonist naloxone. Specifically, morphine-consuming rats showed diarrhea and a significant weight loss ($p < 0.0001$) 30 min after the naloxone injection (Figure 5A,B). Animals also expressed more often the behavioral signs associated with morphine withdrawal, such as wet-dog shakes, forepaw tremors, and chewing ($p < 0.05$) (Figure 5C–E). Additionally, animals showed reduced rearing ($p < 0.001$) during the 30 min observation period after the naloxone injection (Figure 5F), a behavior that is described as a withdrawal sign of orally consumed morphine [20].

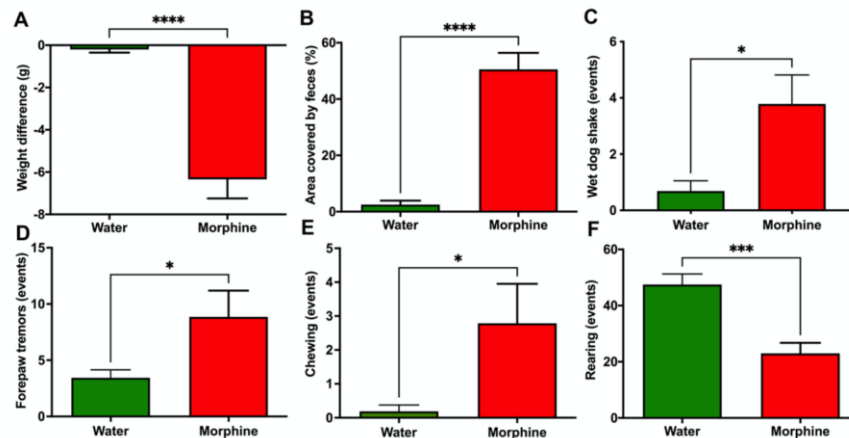


Figure 5. A clear opioid deprivation syndrome is induced by naloxone administration in Wistar rats that had voluntarily consumed morphine. (A) Weight loss, (B) Area covered by feces (%), (C) Wet-dog shake events, (D) Forepaw tremors, (E) Chewing, and (F) Rearing events. Rats that had voluntarily ingested a morphine solution (15–18 mg/kg/day) for four weeks were intraperitoneally injected with 5 mg/kg of the opioid receptor antagonist naloxone. Animals drinking only water were used as controls. Data were recorded for 30 min after naloxone administration and expressed as mean $\pm$ SEM. $n = 8$ in each experimental group. * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$, Two-tailed Student t -test.

2.3. Brain Changes in Rats That Voluntarily Consumed Morphine

To study whether rats that had voluntarily consumed morphine displayed brain neurochemical alterations, we determined the presence of neuroinflammation, oxidative stress levels, and morphine-related protein expression. *Hippocampal neuroinflammation:* Neuroinflammation was evaluated by determining glial reactivity in the hippocampus, assessed as morphological changes in astrocytes and microglial density. Data show that a time period of four weeks of voluntary morphine intake induces a significant increase ($p < 0.0001$) in the length and thickness of hippocampal astrocyte processes (GFAP immunofluorescence) versus those of control animals drinking water (Figure 6A,B). Chronic morphine intake also led to a significant increase ($p < 0.001$) in microglial density (Iba-1 immunofluorescence) compared to control animals (Figure 6A,C).

Hippocampal oxidative stress levels. Oxidized glutathione (GSSG)/reduced glutathione (GSH) levels were assessed in the hippocampus of rats that consumed morphine voluntarily and proper controls to determine the GSSG/GSH ratio, as a validated marker of oxidative stress [23,24]. Data show a significant five-fold increase ($p < 0.001$) in the GSSG/GSH ratio in the hippocampus of morphine-consuming rats compared to that of water-drinking rats (Figure 7).

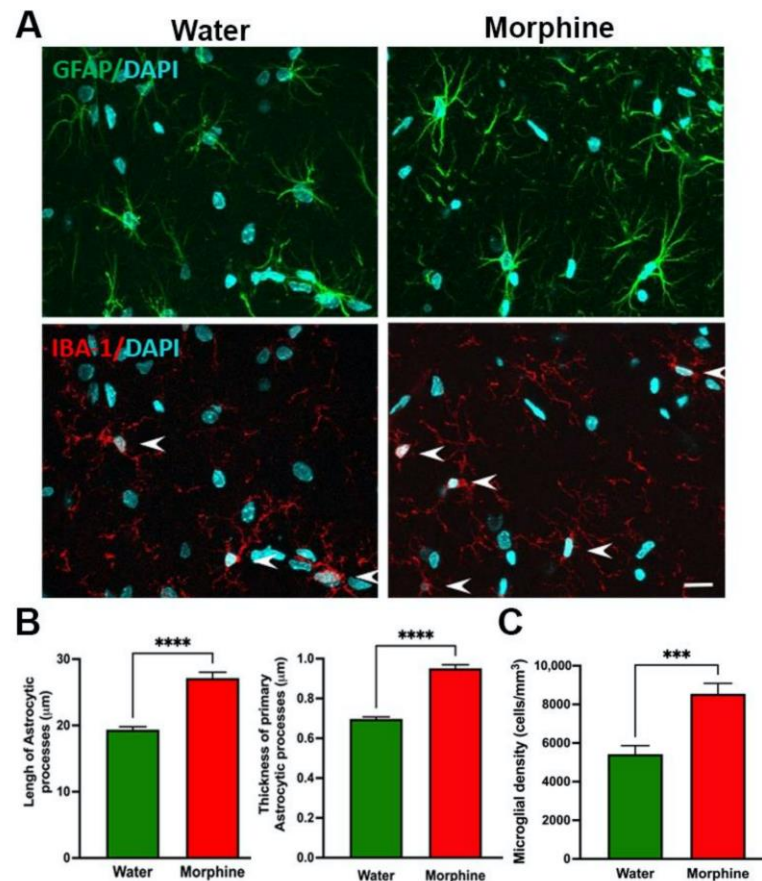


Figure 6. Voluntary morphine intake induces hippocampal neuroinflammation in Wistar rats. (A) Representative confocal microphotographs of hippocampal astrocyte GFAP immunoreactivity (green, top) and microglial density (Iba-1, red, shown by arrows, bottom) double-labeling immunoreactivity of rats which had voluntarily drank morphine solution (15–18 mg/kg/day) for four weeks. Animals drinking only water were used as controls. Nuclei were counterstained with DAPI (blue, nuclear marker), scale bar 25 μm. (B left) Quantification of total length and (B right) thickness of primary astrocytic process and (C) Quantification of microglial density evaluated by confocal microscopy and FIJI image analysis software. Data are expressed as mean ± SEM. $n = 8$ in each experimental group. *** $p < 0.001$, **** $p < 0.0001$, two-tailed Student t -test.

Morphine-related signaling pathway. We observed a significant ($p < 0.01$) increase in the levels of μ -opioid receptor mRNA transcript 1 (OPRM1) in the pre-frontal cortex of rats that consumed morphine voluntarily compared to water-drinking rats (Figure 8). A significant increase ($p < 0.05$) in the pre-frontal cortex was also observed for the mRNA of the accessory protein Regulator of G Protein Signaling 17 (RGS17), but not for the accessory protein vasoactive intestinal peptide (VIP), which are proteins responsible for the modulation of opioid signaling [25,26] (Figure 8). On the other hand, no differences were observed in the nucleus accumbens in the mRNA levels of these proteins, suggesting that the regulation in the mRNA levels of OPRM1 and RGS17 is region-specific.

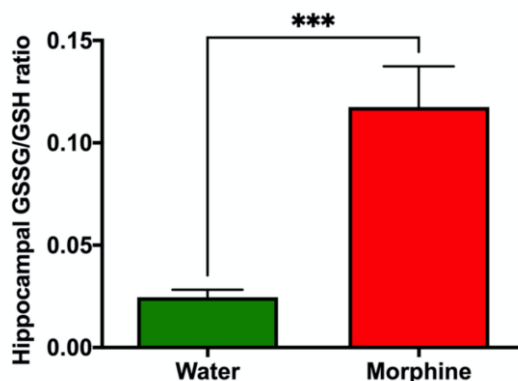


Figure 7. Voluntary morphine intake induces hippocampal oxidative stress in Wistar rats. Hippocampal oxidized/reduced glutathione ratio (GSSG/GSH) in rats that had voluntarily ingested morphine for four weeks. Animals drinking only water were used as controls. Data are expressed as mean $\pm$ SEM. $n = 8$ in each experimental group. *** $p < 0.001$, two-tailed Student t -test.

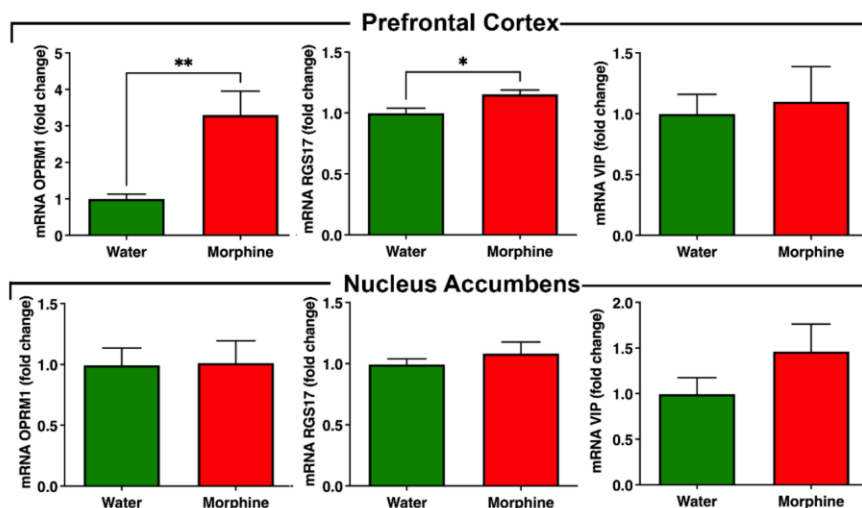


Figure 8. Voluntary morphine intake induces an increase in opioid receptor mRNA levels in pre-frontal cortex but not in nucleus accumbens of Wistar rats. mRNA levels of the morphine receptor OPRM1 and the accessory proteins RGS17 and VIP were determined by RT-qPCR in pre-frontal cortex and nucleus accumbens of rats that had voluntarily ingested morphine solutions for four weeks. Animals drinking only water were used as controls. Expression levels were determined by the $\Delta\Delta CT$ method and normalized against the mRNA levels of the housekeeping genes GAPDH and Hprt1 in the same sample. Data are expressed as mean $\pm$ SEM. $n = 8$ in each experimental group. * $p < 0.05$; ** $p < 0.01$, two-tailed Student t -test.

3. Discussion

Opioid dependence is mostly initiated by the oral administration of prescription opioids, and over 90% of abusers of prescription opioids report their oral ingestion for non-medical purposes [5,6]. While several models of intravenous opioid self-administration are reported [27,28], these do not fully reflect the changes that result from the pharmacokinetic of oral opioid administration. Thus, the generation of a novel and reliable animal model of voluntary oral opioid intake resulting in marked dependence, as shown by a clear

naloxone-induced precipitated withdrawal, would constitute a valuable pre-clinical tool to evaluate new pharmacological strategies for the treatment of oral opioid dependence.

The main limitation in the development of an animal model of oral morphine intake is its bitter taste. Thus, the aim of the present study was to train rats to accept the taste of morphine. In rodents, learning taste preference occurs primarily at weaning time and can influence food selection later in life [17]. It was postulated that repeated exposure to a known aversive flavor at a young age could improve animal flavor acceptance in adult life [18]. Indeed, exposure of rats immediately after weaning to the bitter taste of a quinine solution as the only source of fluid for seven days significantly increases subsequent quinine preference [19]. This was confirmed in the present study since, after only two days of access to a quinine solution as the only source of fluid, rats ingested the quinine solution at the same rate as rats that were allowed water access only. Following seven days of quinine solution intake, rats were able to drink both quinine and morphine solutions, while preferring the morphine solution and further preferring morphine over water, reaching an 80% preference for the morphine solution versus water. These findings suggest that exposure to quinine after weaning renders morphine more palatable.

Although we did not explore the pharmacological effect of quinine consumption during the seven-days period of exclusive consumption of just-weaned rats, we do not expect persisting alterations that may confound the findings associated with the morphine consumption. In that period, rats consumed quinine 30 mg/kg/day, which is comparable to the dose used as an anti-malarian drug in humans. In patients following that regime, mild and reversible adverse reactions are common. Cichonism, observed in most patients treated with quinine 30 mg/kg/day, is comprised of nausea, headache, tinnitus, hearing impairment, and blurred vision [29]. In our study, morphine consumption against water was analyzed 15 to 28 days after rats were drinking high doses of quinine, ensuring the disappearance of the possible adverse effects of quinine exposure.

A limitation of our model is the number of subjects required to be removed from the study. Because our goal was to develop a robust model that could be used to study therapeutics to reduce morphine consumption of dependent subjects, we rejected animals that did not show a preference for morphine against the alternative bottle. Of those rejected, 13% (four animals) were removed after the morphine bottle was given to the rats, which consumed quinine exclusively until then. This suggests that the morphine and quinine solutions were similarly aversive, representing a preference before the animals became dependent. Seven additional rats were removed from the study when the quinine bottle was replaced by one containing only water, since those animals changed their preference from morphine to water. Thus, 18 rats (62% of the starting group of rats) preferred morphine for the complete extension of the study. These rejection rates are in concordance to what was previously reported for other drugs since a diversity in the willingness to consume a drug is observed when outbred animals, i.e., not selectively bred to self-administer a drug, are used as a model for the study of consumption of ethanol [30,31].

Another limitation of our model is the specific age requirement for starting experiments, that being at weaning age, which results in the experiments being performed using young rats. Animals were eight weeks old when euthanasia was performed. On the other hand, our model could be useful for the study of morphine consumption in young individuals, an age segment with high prevalence. In humans, the average age of opioid initiation is between 22 and 23 years old, and teenagers report high availability of illicit opioids [32].

Other research groups also show that morphine is voluntarily consumed by animals when quinine is given in the alternative bottle. However, they show a marked reduction in morphine consumption when quinine is reduced or removed from the alternative bottle [11,15], which suggests that the rewarding/reinforcing effect of morphine (and an induced dependence) was not the main cause of their increased intake.

In the present study, we show that after four weeks of voluntary morphine intake (versus water in controls), the classical acute effects and chronic dependence on morphine

effects are observed, including: (i) thermal and mechanical analgesia; (ii) morphine-induced hyperlocomotion, and importantly (iii) naloxone precipitated withdrawal. The later included (a) significant diarrhea, (b) marked weight loss, (c) wet-dog shakes, (d) forepaw tremors, (e) chewing, and (f) reduced rearing, as previously reported [20,21].

Other studies also aimed at developing animal models for oral opioid self-administration. It is shown that mice [33,34] and rats [35] voluntarily consume oxycodone. In studies in mice, a period of forced oxycodone intake was initially imposed under post-prandial conditions (in the early dark cycle) which was followed by operant self-administration oxycodone intake conditioned by light and tones. In the studies using rats, the animals were initially water deprived for 22 h a day and exposed to oxycodone solutions as their only fluid, also followed by operant self-administration. While, in all these studies, the animals learned to self-administer oxycodone orally, a naloxone precipitated withdrawal was not reported under these oral self-administration conditions. Indeed, in studies using mice, authors induced the naloxone precipitated withdrawal in animals that were administered oxycodone systemically, rather than orally. In contrast, in the rat study of oral operant self-administration of oxycodone, a high dose of naltrexone was not reported to generate the signs of opioid withdrawal. Data suggests that means to induce adult animals to consume oxycodone orally do not readily result in consumption levels that lead to the development of clear physical dependence.

In the present studies, molecular markers of opioid-induced dependence were examined, including higher levels of μ -opioid receptor and RGS17 mRNA levels in the pre-frontal cortex. The latter is a regulator of G-protein signaling that directly interacts with the μ -opioid receptor and promotes opioid tolerance [36]. Increased levels of RGS17 mRNA are associated with morphine preference [36,37]. These alterations were not observed in the nucleus accumbens, suggesting that they are region-specific.

An animal model for voluntary morphine consumption should also reproduce relevant molecular and histological brain alterations that are commonly observed in human patients after morphine exposure. Morphine treatment is shown to promote a rise in brain oxidative stress by both a direct increase of free radicals' levels and a reduction in the enzymatic antioxidant machinery [38], and increases neuroinflammation by the direct activation of Toll-like receptors [39], the hippocampus being the most affected brain region. These phenomena are shown to characterize opioid tolerance and may represent the engines of relapse [38,40]. Indeed, the administrations of antioxidant and anti-inflammatory agents are shown markedly to reduce morphine withdrawal [41] and conditioned place preference [42]. In general, antioxidant and anti-inflammatory agents are shown to reduce the self-administration and relapse of many drugs of abuse, supporting the hypothesis that brain oxidative stress and neuroinflammation are required for an addiction to develop [43]. In the present oral morphine intake model, molecular markers of morphine-induced dependence were clearly observed, including a significant increase in morphine-induced neuroinflammation evidenced by an increase in the length and thickness of primary astrocytic processes and an increase in microglial density in the hippocampus. As expected, morphine-induced neuroinflammation was also accompanied by a significant increase in brain oxidative stress evidenced by an elevated GSSG/GSH ratio in the hippocampus.

Conclusions

Overall, studies conducted show a novel two-bottle choice paradigm to induce a preference for morphine-containing solutions, that starts with young weaning rats, with no influence of taste adulterant confounders nor the need for the animals to consume it to avoid an externally imposed punitive condition. These animals voluntarily drink morphine-containing solutions in amounts that lead to clear morphine dependence, as seen by the classical naloxone-induced signs, and display the molecular and histological brain alterations commonly observed in opioid addicted patients. Thus, this animal model could be valuable for the testing of new therapeutic interventions for the treatment of this devastating and extending condition.

4. Materials and Methods

4.1. Animals

Three-week-old female Wistar rats were used in the experiments. Female rats were chosen since it is reported that female rats consume or self-administer higher levels of morphine than male rats [44–46]. Rats were single-housed at a constant temperature and humidity, on a 12 h light/dark (normal) cycle and with unrestricted access to standard chow and environmental enrichment. All animal procedures were approved by the Committee for Experiments with Laboratory Animals of the Faculty of Medicine of the Universidad del Desarrollo (Protocol 05/2020).

4.2. Voluntary Morphine Consumption Test: Free-Choice Drinking Paradigm

Immediately after weaning, three-week-old rats were randomly assigned to one of two groups. One group was exposed to 0.15 mg/mL quinine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) dissolved in tap water as their only fluid for consumption to accustom the animals to a bitter taste ($n = 29$) [19]. Animals in the second group were only exposed to water (control group) ($n = 8$). Quinine intake, water intake, and body weight were recorded daily. Seven days after starting quinine exposure, animals were changed for two weeks to a two-bottle choice paradigm in which one bottle contained 0.15 mg/mL quinine hydrochloride dissolved in tap water, and the other bottle contained the bitter 0.15 mg/mL morphine sulfate (Oramorph; Molteni Farmaceutici, Scandicci, Florence, Italy) dissolved in tap water. Thereafter, the quinine bottle was removed, and animals were exposed to 0.15 mg/mL morphine sulfate and water (two-bottle choice) for two additional weeks. Animals drinking only water were maintained as controls. Morphine intake, morphine preference, total fluid consumption, and animal weights were measured daily (Figure S1). For two-bottle choice tests, the position of each bottle was alternated daily to prevent the accustoming of the animal to drinking from the bottle of a specific position.

Criteria for subject removal from the study: To develop a robust model to study therapeutics that could reduce morphine consumption, we defined the minimal morphine preference over the alternative bottle to be >50% in volume consumed. Thus, animals that kept a morphine preference below 50% for two consecutive days were removed. There were two instances when subjects were removed: (i) when a morphine bottle was presented to rats drinking only quinine and (ii) when the quinine bottle was replaced with a water bottle.

4.3. Plasma Morphine Determination

After four weeks of voluntary morphine intake, rats were anesthetized by inhalation of 4% sevoflurane vapor (Baxter, Deerfield, IL, USA) in oxygen, and blood samples were collected by cardiac puncture during the first hour of light to expect the highest plasmatic morphine level, since rats mainly drink overnight. Morphine level was determined in the plasma by ELISA using the Morphine ELISA kit (Cayman Chemical, Ann Arbor, MI, USA) following the manufacturer's instructions. Methanol extracted samples were vacuum dried, resuspended in the provided buffer, and 1000-fold diluted before the analysis.

4.4. Evaluation of Morphine-Induced Analgesia

Following four weeks of voluntary morphine intake, analgesia was assessed by the determination of thermal and mechanical sensitivity. For thermal sensitivity determinations, the rats were placed for 20 min inside an acrylic box provided with a mobile on-off infrared light (Ugo Basile Plantar Test) below the surface of the box. Thereafter, the infrared light was placed beneath the mid-plantar surface of a hind paw, and the paw withdrawal response was automatically recorded. The maximum exposure was set at 15 s to avoid hind paw damage. The infrared light exposure was performed to either paw until completing three measurements, with an interval of five minutes between stimuli, and measurements were repeated the following day. The complete control group ($n = 8$) and a random sample of 8 animals from the morphine consumption group were assayed. Data in seconds

were expressed as the mean withdrawal latency registered each day as we previously reported [47].

For mechanical sensitivity determination, animals were placed inside an acrylic box with a mesh floor that allows free access to the plantar surface of the paw. Then, the mid-plantar surface of one of the hind paws was stimulated with an electronic Von Frey filament (Electronic Von Frey; Ugo Basile, Comerio, Varese, Italy) with increasing strength, and the paw withdrawal response was automatically recorded. The stimulation was performed to either paw until completing three measurements, with an interval of five minutes between stimuli, and measurements were repeated the following day. The complete control group ($n = 8$) and a random sample of 8 animals from the morphine consumption group were assayed. Thermal and mechanical sensitivities were measured in a 23 °C controlled temperature room during the first hour of light to expect the highest plasmatic morphine levels.

4.5. Evaluation of Morphine-Induced Locomotor Activity

Following four weeks of voluntary morphine intake, locomotor activity was assessed by the open field test. The open field consisted of a square base (100 × 100 cm) and walls of 40 cm made of black painted wood. Rats were placed in the center, and their locomotor activity was recorded for five minutes with a video camera placed in a zenithal position. The open field arena was cleaned with 70% ethanol after each animal session. The room was lit by a soft white light (50–60 lux). The experiments were performed during the first hour of light to expect the highest plasmatic morphine levels. Total distance traveled and thigmotaxis time (tendency to remain close to the walls) were evaluated using the ANY-maze video tracking system (ver. 6.35, Stoelting Co., Wood Dale, IL, USA). The complete control group ($n = 8$) and a random sample of 8 animals from the morphine consumption group were assayed.

4.6. Induction of Withdrawal Syndrome

For induction of the withdrawal syndrome, rats that had voluntarily ingested the morphine solution for four weeks were intraperitoneally injected 5 mg/kg of the opioid receptor antagonist naloxone (Sigma-Aldrich) dissolved in 0.9% saline. Immediately after naloxone administration, animals were placed in a glass beaker (300 mm height and 180 mm diameter) and monitored for weight loss, diarrhea, wet-dog shakes, forepaw tremors, chewing, and rearing events over 30 min. The complete control group ($n = 8$) and a random sample of 8 animals from the morphine consumption group were assayed.

4.7. Evaluation of Morphine-Induced Neuroinflammation

Neuroinflammation was evaluated, determining astrocyte activation and microglial density in the hippocampus of rats after four weeks of voluntary morphine intake. Animals were anesthetized by inhalation of 4% sevoflurane vapor in oxygen (Baxter), intracardially perfused with 100 mL of PBS (pH 7.4), and euthanized to obtain brain samples. Double-labeling immunofluorescence against the astrocyte marker glial fibrillary acidic protein (GFAP) and the microglial marker ionized-calcium-binding adaptor molecule 1 (Iba-1) were evaluated in coronal 30 mm thick cryo-sections of hippocampus as we previously reported [48]. Briefly, coronal sections were washed with 0.1 M PBS, incubated with blocking solution (0.3% Triton X-100, 0.1% BSA, and 10% normal goat serum in PBS) for 1 h, followed by incubation with a primary rabbit monoclonal anti-IBA-1 antibody (cat#019-19741, Wako, 1:500 dilution in blocking solution) at 4 °C overnight. Thereafter, sections were rinsed in PBS containing 0.3% Triton X-100 and incubated with goat anti-rabbit secondary antibody (Alexa Fluor 594, Thermo Fisher Scientific, Waltham, MA, USA; 1:500 dilution in blocking solution) for 1 h in the dark at room temperature. Then, sections were rinsed, incubated in blocking solution (0.3% Triton X-100, 1% BSA, and 5% normal goat serum in PBS) for 1 h and subsequently with a primary mouse monoclonal anti-GFAP antibody (cat#G3893, Sigma-Aldrich, 1:500 dilution in blocking solution) overnight at 4 °C in the

dark. Samples were rinsed with PBS, incubated with goat anti-mouse secondary antibody (Alexa Fluor 488, Thermo Fisher Scientific, 1:500 dilution in blocking solution) for 1 h in the dark at room temperature. Nuclei were counterstained with DAPI (Invitrogen, Waltham, MA, USA; 0.02M: 0.0125 mg/mL). Microphotographs were taken from the stratum radiatum of the hippocampus using a confocal microscope (Olympus FV10i). The area analyzed for each stack was 0.04 mm², and the thickness (Z axis) was measured for each case. The total length and thickness of the GFAP-positive astrocyte primary process and density of Iba-1-positive microglial cells were assessed using FIJI image analysis software as previously reported [24]. The complete control group ($n = 8$) and a random sample of 8 animals from the morphine consumption group were assayed.

4.8. Evaluation of Morphine-Induced Oxidative Stress

Brain oxidative stress was evaluated, determining the oxidized glutathione (GSSG)/reduced glutathione (GSH) ratio (GSSG/GSH) in the hippocampus of rats after four weeks of voluntary morphine intake. To this end, the hippocampi were extracted and mixed with three volumes of ice-cold potassium buffer (0.1M) containing 5 mM EDTA, pH 7.4. GSH and GSSG contents were determined as previously described [24]. Briefly, GSSG in the sample was first converted into GSH with glutathione reductase (Sigma-Aldrich) and NADPH (Sigma-Aldrich). The total free thiol group of GSH was reacted with the sulfhydryl reagent DTNB (Sigma-Aldrich), yielding a product that absorbs light at 412nm. GSSG per se in the homogenate was measured by adding 2-vinyl pyridine (Sigma-Aldrich) to trap GSH, preventing GSH from binding DTNB. The excess of 2-vinyl pyridine was neutralized with triethanolamine. Finally, the free GSH levels were obtained by subtracting the GSSG value to total GSH. The complete control group ($n = 8$) and a random sample of 8 animals from the morphine consumption group were assayed.

4.9. Determination of mRNA Levels of Morphine Receptor and Accessory Proteins

Four weeks after voluntary morphine intake, total RNA was isolated from the nucleus accumbens and pre-frontal cortex using TRIzol reagent (Invitrogen) following manufacturer's instructions. One microgram of total RNA was used to perform reverse transcription with MMLV reverse transcriptase (Invitrogen) and oligo dT primers. Real-time PCR reactions were performed in a 10 mL final volume containing: 50ng cDNA, PCR LightCycler-DNA Master SYBERGreen reaction mix (Roche, Basel, Switzerland), 3 mM MgCl₂ and 0.5mM of the primers for the amplification of the opioid receptor OPRM1, and the accessory proteins RGS17 and VIP using a Light-Cycler 1.5 thermocycler (Roche) as previously reported [23]. To ensure that amplicons were generated from mRNA and not from genomic DNA, controls without RT during the reverse transcription reactions were included. Expression levels were determined by the $\Delta\Delta C_t$ method. The mRNA level of each target gene was normalized against the mRNA levels of the housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyltransferase 1 (Hprt1) in the same sample. The primers used for qPCR reactions were: OPRM1 forward 5'-ATCCAGTTCTTTACGCCTTCC-3'; OPRM1 reverse 5'-GATGTTCCCTAGTGTTCTGA CG-3'; RGS17 forward 5'-GAACAGAATACAGCGAGGAGAA-3'; RGS17 reverse 5'-GACCTCTTTCGGTGACAGTATAG-3'; VIP forward 5'-CATTGGCAAACGAATCAG CA GT-3'; VIP reverse 5'-CTCACTGCTCCTCTTCCCATTAG-3'; GAPDH forward 5'-GACATGCCGCCTGGAGAAAC-3'; GAPDH reverse 5'-AGCCCAGGATGCCCTTAGT-3'; Hprt1 forward 5'-CTGGTGAAAAGGACCTCTCG-3'; Hprt1 reverse 5'-TCCACTTTCGCTGAT GACAC-3'. The complete control group ($n = 8$) and a random sample of 8 animals from the morphine consumption group were assayed.

4.10. Statistical Analysis

Statistical analyses were performed using GraphPad Prism software. Data are expressed as mean $\pm$ SEM. Two-way (treatment $\times$ day) analysis of variance (ANOVA), followed by the Bonferroni post hoc test, was conducted to compare liquid intake. Stu-

dent's *t*-test was used to determine if two sets of data were significantly different from each other. A level of $p < 0.05$ was considered for statistical significance. GraphPad Prism version 9.2.0 was used for statistical analysis. To facilitate text reading, the full statistical analyses are presented in the figure legends.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/ijms23073874/s1>.

Author Contributions: P.B.-C. contributed to the conception and design, collection of data, data analysis, manuscript writing, and final approval of the manuscript. M.Q., D.S., P.M., B.O., C.P., A.Á., C.D.G., M.E., M.E.Q. and M.H.-M. conducted the collection of data, data analysis, and final approval of the manuscript. Y.I. contributed to data analysis, manuscript writing, and final approval of the manuscript. F.E. contributed to the conception and design, collection of data, data analysis, financial support, manuscript writing, and final approval of the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by FONDECYT 1200287 and ANID ACT210012 to Fernando Ezquer and FONDECYT 3210276 to Pablo Berrios-Cárcamo.

Institutional Review Board Statement: All animal procedures were approved by the Committee for Experiments with Laboratory Animals of the Medical Faculty of the Universidad del Desarrollo (Protocol 05/2020).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are available upon request.

Acknowledgments: The technical assistance of Camila Ezquer, Jorge Ruiz, and Catalina Vallejos is greatly appreciated.

Conflicts of Interest: The authors declare no conflict of interest.

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5. Chapter 2

Effect of human mesenchymal stem cell secretome administration on morphine self-administration and relapse in two animal models of opioid dependence

Access: <https://www.nature.com/articles/s41398-022-02225-0>

ARTICLE OPEN



Effect of human mesenchymal stem cell secretome administration on morphine self-administration and relapse in two animal models of opioid dependence

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The present study investigates the possible therapeutic effects of human mesenchymal stem cell-derived secretome on morphine dependence and relapse. This was studied in a new model of chronic voluntary morphine intake in Wistar rats which shows classic signs of morphine intoxication and a severe naloxone-induced withdrawal syndrome. A single intranasal-systemic administration of MSCs secretome fully inhibited (>95%; $p < 0.001$) voluntary morphine intake and reduced the post-deprivation relapse intake by 50% ($p < 0.02$). Since several studies suggest a significant genetic contribution to the chronic use of many addictive drugs, the effect of MSCs secretome on morphine self-administration was further studied in rats bred as high alcohol consumers (UChB rats). Sub-chronic intraperitoneal administration of morphine before access to increasing concentrations of morphine solutions and water were available to the animals, led UChB rats to prefer ingesting morphine solutions over water, attaining levels of oral morphine intake in the range of those in the Wistar model. Intranasally administered MSCs secretome to UChB rats dose-dependently inhibited morphine self-administration by 72% ($p < 0.001$); while a single intranasal dose of MSC-secretome administered during a morphine deprivation period imposed on chronic morphine consumer UChB rats inhibited re-access morphine relapse intake by 80 to 85% ($p < 0.0001$). Both in the Wistar and the UChB rat models, MSCs-secretome administration reversed the morphine-induced increases in brain oxidative stress and neuroinflammation, considered as key engines perpetuating drug relapse. Overall, present preclinical studies suggest that products secreted by human mesenchymal stem cells may be of value in the treatment of opioid addiction.

Translational Psychiatry (2022)12:462; <https://doi.org/10.1038/s41398-022-02225-0>

INTRODUCTION

Opioid dependence has become a public health emergency, with over 16 million opioid-dependent individuals worldwide [1, 2]. In 2022 the CDC's National Center for Health Statistics reported an estimated 107,622 opioid overdose deaths in the United States during 2021, an increase of nearly 15% from the 93,655 deaths estimated in 2020. The situation is also dire in other countries; the World Health Organization (WHO 2021) [3] reports that over one half million deaths are attributable to drug use, and over 70% of these deaths are related to opioids. Thus, opioid addiction has become major cause of preventable morbidity and mortality worldwide. Recently, Harper et al. [4] informs that life expectancy in the United States had three consecutive years of decline. Among the causes for this decline the first one indicated is the increase in opiate overdose deaths. Noteworthy, a recent study further indicates that the increase in overdose mortality is explained largely by the availability and relatively low cost of opioids rather than by economic conditions [5].

Currently available treatments for opioid dependence mainly involve the administration of long-acting opioid agonists, such as methadone or buprenorphine, which have shown temporary efficacy in reducing illegal opioid use [6]. However, these substitution therapies maintain opioid dependence, leading to high rates of relapse upon treatment discontinuation [7]. Therefore, there is an urgent need to develop novel non-opioid-based pharmacological approaches.

Several preclinical and clinical studies have shown that brain oxidative stress and neuroinflammation are generated following the chronic administration of most drugs of abuse, including morphine, and are strongly associated with the perpetuation of drug intake and relapse [8–10]. Morphine consumption induces oxidative stress by the release of dopamine in the nucleus accumbens, which is oxidized by monoamine oxidase, generating hydrogen peroxide and hydroxyl radicals after its presynaptic re-uptake [8] and by the NO synthesis after μ -opioid receptor (MOR) activation [11]. Neuroinflammation also results from the chronic use of opioids [12], since morphine binds to the foreign-molecule

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Received: 14 April 2022 Revised: 14 October 2022 Accepted: 19 October 2022
Published online: 04 November 2022

sensor Toll-like receptor 4 (TLR4) in microglia and astrocytes, leading to the initiation of a signaling cascade that results in microglial and astrocytic activation and the production of pro-inflammatory mediators [10, 13, 14].

Morphine activation of MORs in the ventral tegmental area stimulates the reward system by increasing dopamine release into the nucleus accumbens and prefrontal cortex [15], playing a central role in the reinforcing properties of morphine [16]. Importantly, if after chronic use a prolonged drug deprivation is held, environmental cues associated with prior drug use trigger the urge to re-administer the drug, leading to relapse and the perpetuation of drug intake, a phenomenon that can persist for many years [16]. This behavior is driven by an elevation of extracellular glutamate in the tripartite synapse due to a cue-induced glutamate release into the nucleus accumbens [17–19].

Astrocytes surrounding the synapses are responsible for most of the glutamate re-uptake, playing a pivotal role in the termination of glutamatergic signaling [20]. However, this re-uptake is faulty in drug users as high levels of reactive oxygen species (ROS), by cross-linking of cysteine residues, can directly inhibit the main astrocyte glutamate transporter GLT-1 [21], and possibly the xCT cystine-glutamate antiporter [22], a carrier involved in glutamatergic downregulation. Neuroinflammation per se also lowers astrocyte GLT-1 levels and increases the extracellular glutamate levels [23]. Importantly, oxidative stress and neuroinflammation self-potentiate each other [24], being linked in a *long-acting vicious cycle* that is maintained in the brain of opioid users, altering glutamate homeostasis [8]. Therefore, interventions aimed at restoring glutamate homeostasis by the reduction of morphine-induced oxidative stress and neuroinflammation could help to reduce morphine dependence.

In recent years, mesenchymal stem cells (MSCs), an adult type of stem cell, have emerged as promising candidates for the treatment of neurological diseases commonly associated with neuroinflammation and oxidative stress [25, 26]. These cells have potent immunomodulatory properties, mainly mediated by the secretion of anti-inflammatory molecules which promote the inhibition of astrocyte and microglia activation [27], and by the production of high levels of antioxidant enzymes [28], which contribute to reduce oxidative stress. These anti-inflammatory and antioxidant properties are greatly enhanced by incubating MSCs with pro-inflammatory cytokines; a process known as preconditioning [29, 30].

Recently, it was shown that a single intra-cerebroventricular administration of rat MSCs [31] or human preconditioned MSCs [30] to rats that had consumed alcohol chronically reduced their voluntary ethanol intake by 75% and inhibited alcohol relapse upon re-access up to 85%. These effects were correlated with a significant reduction of neuroinflammation and oxidative stress and with an increase in GLT-1 levels in nucleus accumbens [30].

Initially MSCs were thought to exert their therapeutic effects by their multipotent differentiation capacity, replacing damaged cells in injured tissues. However, currently, the paracrine secretion (secretome) of a broad array of bioactive molecules is the most accepted therapeutic mechanism associated with MSCs [32, 33]. In line with this view, it was recently reported that the intranasal administration of cell-free secretome derived from preconditioned human MSCs to alcohol-dependent rats fully recapitulated the therapeutic effects of live MSC administration, including a 90% reduction in chronic alcohol intake, complete prevention of cue-induced alcohol relapse and fully reversion of hippocampal neuroinflammation and oxidative stress [34]. These effects were also shown after the intranasal administration of MSC-derived secretome in an animal model of nicotine dependence [34].

In the present study, using two rat models of oral morphine self-administration, we evaluated whether the administration of secretome derived from human preconditioned MSCs was able to reduce voluntary oral morphine intake and prevent relapse. The

first model, as recently reported [10], required training animals to accept the bitter taste of morphine. For the second one, we used rats selectively bred to consume alcohol (UChB), which also have been shown to voluntarily consume nicotine [34, 35], in which voluntary oral morphine intake was induced after injecting priming doses of morphine. Further, we evaluated whether secretome administration was able to reverse molecular markers associated with opioid relapse, including morphine-induced neuroinflammation and oxidative stress and changes in opioid receptor and glutamate transporters mRNA levels. It is hypothesized that MSC-derived secretome administration will inhibit morphine self-administration and will markedly inhibit post-deprivation relapse.

MATERIALS AND METHODS

Animals

Model 1. Wistar rats. Just-weaned three-week-old female Wistar rats weighing 50–65 g were single-housed at constant temperature on a 12-h light/dark cycle and unrestricted access to standard rodent chow.

Model 2. Wistar-derived UChB rats. Eight-week-old female naïve rats weighing 95–120 g of the UChB line selectively bred for their high voluntary oral ethanol intake [36, 37] were single-housed at a constant temperature on a 12-h light/dark cycle with unrestricted access to rodent chow and water.

Both animal models were conducted with female rats since oral morphine consumption, or its self-administration is higher in females than in male rats [38–40]. Animal procedures were approved by the Committee for Experiments with Laboratory Animals of the Universidad del Desarrollo (Protocol-05/2020) and by the Committee for Experiments with Laboratory Animals of the University of Chile (Protocol-CBA# 0994-FMUCh).

Voluntary morphine consumption and relapse

Animal model 1 (Wistar rats). Voluntary oral consumption of morphine solution was induced in Wistar rats as recently described, where plasma morphine levels of $2.5 \pm 0.6 \mu\text{g/ml}$ were attained after 4 weeks of voluntary intake [10]. Briefly, just-weaned rats were accustomed to the bitter taste of 150 mg/l quinine hydrochloride (Sigma-Aldrich) dissolved in tap water as their only fluid source available for seven days, after which animals were changed for two weeks to a two-bottle choice paradigm in which one bottle contained 150 mg/l quinine hydrochloride and the other 150 mg/l of the bitter morphine sulfate (Oramorph, Molteni Pharmaceuticals) dissolved in tap water. Thereafter, animals were offered free-choice access between two bottles, one containing water and the other 150 mg/l morphine sulfate for two additional weeks. After 22 days of voluntary morphine intake animals were randomly divided into two groups that received a simultaneous intranasal (25 μg protein) and intravenous (25 μg protein) dose of secretome derived from 1×10^6 preconditioned MSCs ($n = 9$) or saline ($n = 9$) administered in a volume of 160 μl . An additional group of animals drinking only water was used as control. To evaluate morphine post-deprivation relapse, in the same group of rats after 30 days of chronic morphine intake animals were morphine deprived for 12 days (water was available) and on days 2 and 9 of the deprivation period rats were treated with two simultaneous intranasal and intravenous administrations of secretome derived from preconditioned MSCs or vehicle. At the end of the deprivation period, animals were allowed morphine re-access for 24 hours to assess relapse, after which animals were euthanized (Supplementary Fig. 1A shows the time-course of experiment with Wistar rats).

Animal model 2 (UChB rats). Voluntary oral morphine consumption was induced in UChB rats as previously described [41, 42] with some modifications. Briefly, rats were intraperitoneally administered a daily dose of 40 mg/kg of morphine hydrochloride (Sanderson Laboratory) in a volume of 2 ml/kg/day for 9 consecutive days. On day 10, after discontinuation of morphine injections, all rats were given free-choice access to two bottles: one containing water and the other morphine sulfate solution (Oramorph) of concentrations that were increased every two to four days, from 6 mg/l and reaching a steady intake of 50 mg/l on days 40 to 58. Thereafter, rats were given concurrently free choice access between three bottles: one with water and the other two containing 44 and 50 mg/l morphine sulfate from day 59 to 89. After 10 weeks of

voluntary oral morphine consumption, animals were randomly divided into two groups ($n=4$) that received an intranasal dose (25 µg protein) of secretome derived from 1×10^6 preconditioned MSCs or saline, administered in a total volume of 160 µl/day, on days 75, 80 and 85. Thereafter, to evaluate morphine post-deprivation relapse, four days after the last intranasal secretome dose animals were deprived of the morphine solutions for five days and were administered a fourth dose of secretome or saline on the first day of the deprivation period. Subsequently, rats were allowed re-access to the 44- and 50 mg/l morphine solutions for one day.

To separately evaluate the effects of secretome on morphine relapse, secretome was intranasally administered only during the deprivation period to another group of sixteen UChB rats that were subjected to the same paradigm described for the first UChB group, but slightly modified to increase voluntary morphine intake and to induce a more potent morphine relapse. Rats were administered an intraperitoneal daily dose of 40 mg/kg of morphine hydrochloride in a volume of 2 ml/kg/day for 11 consecutive days. After discontinuation of morphine injections on day 12, rats were offered a free-choice between a morphine solution of increasing concentration on successive days (6 to 90 mg/l morphine) or water until day 42. Thereafter, rats were given concurrently free-choice access between three bottles: one containing water and the other two containing 80 or 90 mg/l morphine sulfate solutions from day 43 to 57. After eight weeks of continuous voluntary morphine consumption, animals were deprived of morphine for six days and on the fourth day of morphine deprivation, animals were randomly divided into 2 groups ($n=6$) and were treated with a single intranasal dose of secretome (25 µg protein) derived from 1×10^6 preconditioned MSCs or saline administered in a volume of 160 µl. Rats continued under morphine deprivation for two additional days and thereafter animals were allowed re-access to the 80- and 90 mg/l morphine solutions for two days (Supplementary Fig. 1B and C show the time-course of experiments with UChB rats).

In both animal models, the bottle positions were alternated every day to avoid the development of a side preference. Morphine intake, water intake, and animal body weight were measured daily. In both models reported, morphine is mainly consumed by the animals in the dark period of the circadian cycle.

Isolation, expansion and characterization of human adipose tissue-derived MSCs

Human MSCs were isolated from fresh subcutaneous adipose tissue samples obtained from liposuction aspirates of patients undergoing cosmetic liposuction at Clínica Alemana, Santiago, Chile after obtaining written informed consent as previously reported [34, 43]. Protocols were approved by the Ethics Committee of Faculty of Medicine, Clínica Alemana-Universidad del Desarrollo. After two subcultures, cells were characterized following the minimum criteria established by the International Society for Cellular Therapy [44] as previously described [34, 43].

Preconditioning of MSCs and secretome generation

MSCs (passage 3) at 75% confluency were preconditioned to improve the production of anti-inflammatory and antioxidant molecules by incubation in minimal essential medium (α -MEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Hyclone) plus 10 ng/ml TNF- α (R&D System) and 15 ng/ml IFN- γ (R&D System) for 40 hours as previously described [30, 34, 45]. After preconditioning, cells were washed and incubated for 48 hours in α -MEM without FBS. Then, culture medium (secretome) was collected and centrifuged at 400 g for 10 minutes to remove detached cells. The supernatant was centrifuged again at 5,000 g for 10 minutes to remove cell debris. Finally, secretome was filtered with 0.22 µm filters and concentrated 50 times (v/v) using 3 kDa cutoff filters (Millipore). Protein concentration was determined by the BCA protein assay kit (Thermo Scientific) and secretome was aliquoted and frozen at -80°C until use.

Non-invasive administration of secretome derived from human preconditioned MSCs

- A. **Intranasal administration:** Rats were anesthetized by intramuscular administration of ketamine (60 mg/kg) and acepromazine (4 mg/kg) [46] and kept in supine position. Twenty microliters of secretome solution were administered intranasally as small drops delivered from a pipette tip every five minutes into alternative sides of the nasal cavity (four times in each nostril) for a total of 40 minutes. A

total volume of 160 µl of secretome containing 25 µg of proteins derived from 1×10^6 preconditioned MSCs was delivered into the nasal cavity. Vehicle-treated animals received 160 µl of saline solution by the same administration scheme as previously reported [34].

- B. **Intravenous administration:** In the Wistar rat (Animal Model #1) studies, in addition to the intranasal administration animals received secretome by the intravenous route. Immediately after the intranasal administration, animals were injected via the tail vein with 160 µl of secretome containing 25 µg of proteins derived from 1×10^6 preconditioned MSCs. Vehicle-treated animals received 160 µl of saline solution using the same administration scheme.

Evaluation of morphine-induced neuroinflammation

In both animal models, neuroinflammation was evaluated as previously described [34, 47], determining astrocyte activation and microglial density in the hippocampus of rats after the 24-hours morphine re-access. Briefly, after the last morphine intake register animals were anesthetized by the intramuscular administration of 1.9 ml/kg of an anesthetic cocktail (70 mg/kg ketamine, 10 mg/kg xylazine and 4 mg/kg acepromazine) [48], intracardially perfused with 0.1 M PBS (pH 7.4) and euthanized. Double-labeling immunofluorescence against the astrocyte marker glial fibrillary acidic protein (GFAP) (Sigma-Aldrich G3893, 1:500 dilution) and the microglial marker ionized-calcium-binding adaptor molecule 1 (Iba-1) (Wako 019-19741, 1:400 dilution) were performed in coronal 30 µm thick cryo-sections of hippocampus. Nuclei were labeled with DAPI (Invitrogen). Microphotographs were taken from the *stratum radiatum* of hippocampus using a confocal microscope (Zeiss, LMS700). The area analyzed for each stack was 0.04 mm². The total length and thickness of primary astrocytic processes and the density of microglial cells were determined using FIJI image analysis software as previously reported [34, 47].

Evaluation of morphine-induced oxidative stress

In both animal models, after the 24-hour morphine re-access brain oxidative stress was evaluated determining the ratio between oxidized glutathione/reduced glutathione (GSSG/GSH) in the hippocampus and the levels of lipid peroxidation in the neostriatum. The GSSG/GSH ratio was determined as we previously reported [34, 47]. Lipid peroxidation was determined by a method that measures the amount of malondialdehyde (MDA) formed using the Lipid Peroxidation assay kit (Sigma-Aldrich) as we previously described [49].

Determination of mRNA levels of opioid receptors and glutamate transporters

After the 24-hour morphine re-access, total RNA from prefrontal cortex and nucleus accumbens was purified using TRIzol reagent (Invitrogen). One microgram of RNA was used to perform reverse transcription with MMLV reverse transcriptase (Invitrogen) and oligo dT primers. Real-time PCR reactions were performed to amplify the μ -opioid receptor 1 (OPRM1) and the glutamate transporters GLT-1 and xCT, using specific primers (Supplementary Table 1) and SYBRGreen reagent (Roche) in a LightCycler thermocycler (Roche). Relative quantifications were performed by the $\Delta\Delta\text{CT}$ method. The mRNA level of each target gene was normalized against the mRNA level of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All biochemical analysis were performed in a single-blind way.

Statistical analysis

Data are expressed as means $\pm$ SEM. Statistical analyses were performed using GraphPad Prism v.9.2.0 software. The normal distribution of data for all experiments was shown using the Shapiro-Wilk test. Bartlett's test showed that the variances between groups were equal. Two-way (treatment $\times$ day) ANOVA, followed by Tukey's post-hoc test or One-way ANOVA, followed by Tukey's post-hoc test was conducted to compare differences between experimental groups. Two-tailed Student t test was performed to determine if there is statistical significance when only two groups were compared. A level of $p < 0.05$ was considered for statistical significance.

RESULTS

Model 1: Wistar rats

Simultaneous intranasal and intravenous administration of secretome derived from human preconditioned MSCs to Wistar rats

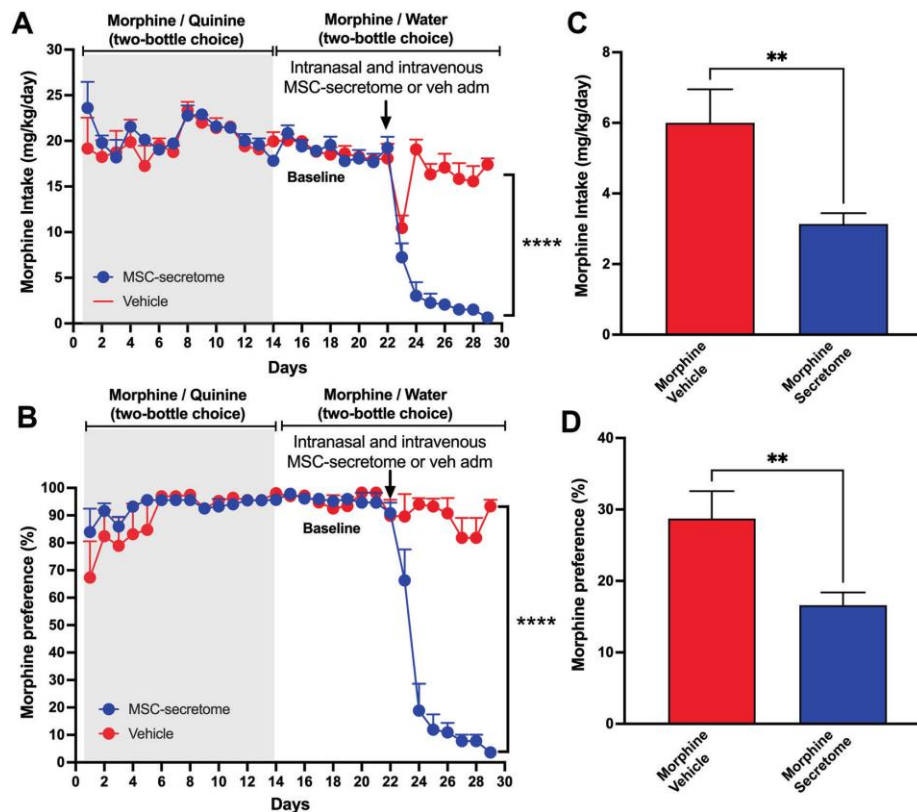
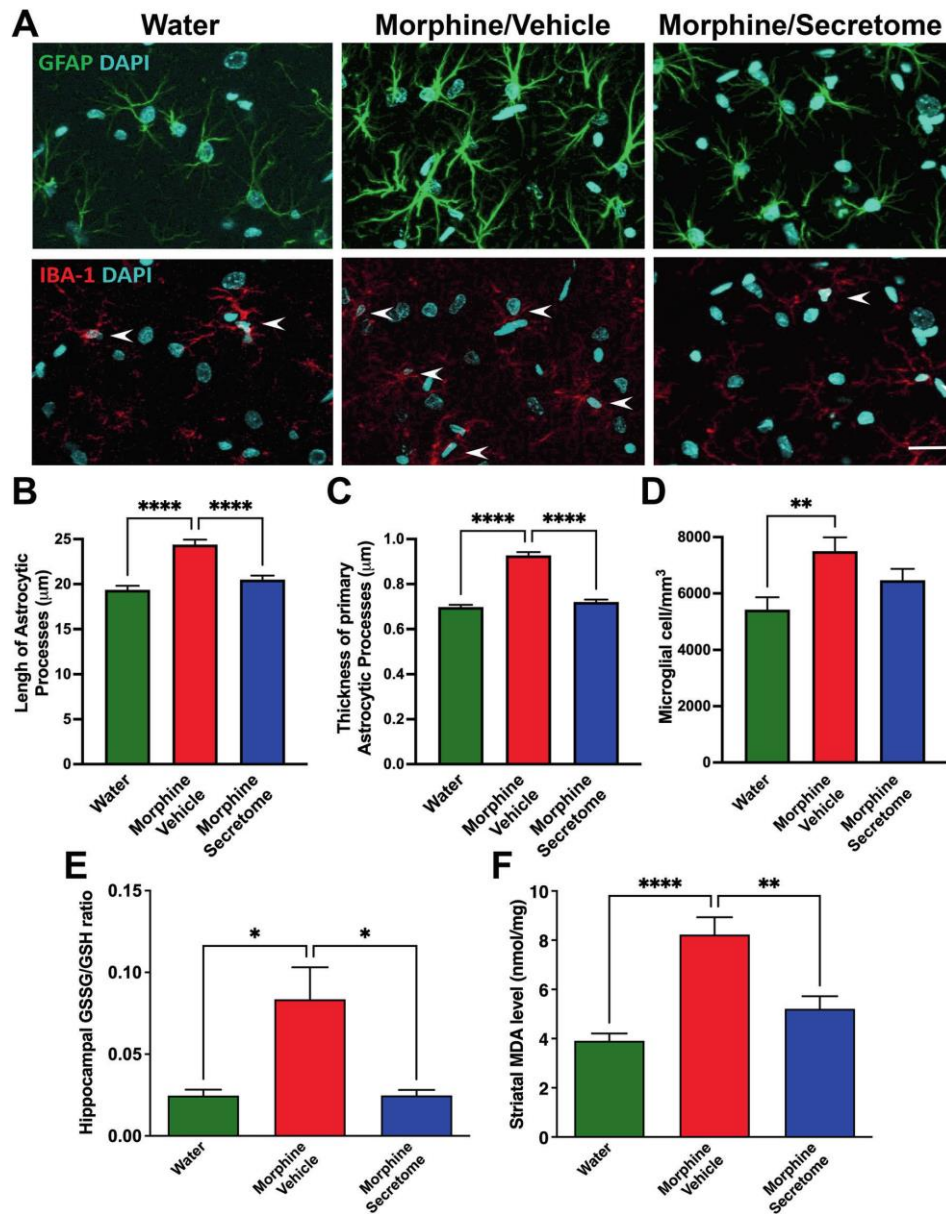


Fig. 1 Simultaneous intranasal and intravenous administration of secretome derived from human preconditioned MSCs to Wistar rats that had chronically consumed morphine inhibits morphine intake and reduces post-deprivation morphine re-access intake. **A** Daily morphine intake and **(B)** morphine preference over water. Wistar rats that had voluntarily ingested morphine for 21 days were simultaneously intranasally and intravenously treated with secretome (25µg protein) derived from 1×10^6 preconditioned MSCs or vehicle (indicated by the arrow). Morphine intake was inhibited by 95% ($p < 0.0001$). Two-way ANOVA (treatment $\times$ day) of morphine intake and morphine preference following secretome administration (blue circles) indicate significant effect of treatment ($F_{(1,435)} = 158.9$ **** $p < 0.0001$), day ($F_{(28,435)} = 30.4$, $p < 0.0001$) and significant interaction ($F_{\text{treatment} \times \text{day}} = 25.2$, $p < 0.0001$) compared with control animals receiving vehicle (red circles). **C** Morphine relapse intake and **(D)** morphine preference over water after 12 days of morphine deprivation followed by 24-hours of morphine re-access. Rats treated previously with three intranasal and intravenous secretome doses (on day 22 of chronic morphine access and on days 2 and 9 of the deprivation period) (blue bars) ingested significantly lower (~50%) amount of morphine compared with vehicle-treated animals (red bar) (** $p < 0.01$, two-tailed Student's t test). Data are presented as mean $\pm$ SEM; $n = 9$ for each experimental condition.

inhibits chronic morphine intake and reduces post-deprivation morphine re-access intake. In this model of opioid dependence [10], just-weaned Wistar rats were exposed for seven days to a quinine solution as their only fluid source to get the animals accustomed to a bitter taste (Fig. 1 day zero), after which animals were offered a two-bottle choice of quinine or morphine solutions, for two weeks. Figure 1A (shaded area) shows that Wistar rats that could choose between a 150 mg/l quinine solution versus one of 150 mg/l morphine achieved a high morphine consumption (20.1 ± 0.4 mg/kg/day) for 14 days. Four animals (15%) kept a significant quinine preference (below 50% preference for the morphine solution) and were removed from the study. Subsequently, animals were offered a two-bottle choice between a morphine solution (150 mg/l) and water from day 15 onwards, showing a voluntary morphine intake of 18.5 ± 0.3 mg/kg/day (Fig. 1A), with a preference for morphine solution over water above 85% (Fig. 1B). As previously reported [10], these levels of morphine intake allow the development of strong naloxone-induced withdrawal signs of dependence. In the present study, five animals

changed their preference ratio from the morphine solution to water and were removed from the study. Thus, the overall rejection corresponds to 9 animals of the original 27 animals (33%), in line with a previous report [10]. After removing the animals that changed morphine preference, the remaining animals were distributed into two groups according to their morphine intake on the last three days, such as to avoid group differences. Prior to initiating the secretome or vehicle administration, the average 3-day intake was 18.1 ± 0.7 mg/kg/day for the animals in the vehicle treated group and 18.3 ± 0.7 mg/kg/day for the animals in the secretome treated group.

To evaluate the therapeutic potential of MSCs, the secretome derived from 1×10^6 preconditioned human adipose tissue-derived MSCs (25µg protein) was administered simultaneously intranasally and intravenously to one half of the rats that had voluntarily consumed morphine for 22 days ($n = 9$), while the other half were given saline (vehicle) as control ($n = 9$). Secretome administration virtually abolished morphine dependence ($p < 0.0001$, Two-way ANOVA) (Fig. 1A), reaching >95% reduction



in morphine intake seven days after the single-time secretome administration compared with vehicle-treated animals (0.6 ± 0.3 mg morphine consumed/kg/day for secretome-treated animals versus 17.4 ± 0.7 mg morphine consumed/kg/day for vehicle-treated animals). Noteworthy, most of the inhibitory effect on morphine intake was seen within the first 48 hours (>80% reduction in morphine intake). Secretome administration also reduced morphine preference versus water ($p < 0.0001$, Two-way ANOVA) (Fig. 1B). The reduction in morphine intake induced by secretome administration was fully compensated by an increase in

water intake (Supplementary Fig. 2A); additionally, secretome administration did not affect animal body weight (Supplementary Fig. 2B), both suggesting that the effects induced by this treatment were specific for morphine intake and do not reflect a nonspecific malaise nor severe withdrawal symptoms.

To evaluate the effect of secretome administration on post-deprivation relapse intake after morphine re-access, seven days after the administration of secretome or vehicle animals were morphine-deprived for 12 days and treated with two intranasal and intravenous administrations of secretome or vehicle (on days 2

Fig. 2 Simultaneous intranasal and intravenous administration of secretome derived from human preconditioned MSCs normalizes the increased astrocyte reactivity and brain oxidative stress induced by chronic morphine intake in Wistar rats. **A** Representative confocal microphotographs of GFAP immunoreactivity (green top) in hippocampal astrocytes and Iba-1 immunoreactivity (red, shown by arrows, center) in hippocampal microglia. Nuclei were counterstained with DAPI (blue, nuclear marker), scale bar 25 μ m. **B** Quantification of length and **(C)** thickness of primary astrocytic processes. **(D)** Quantification of microglial density. Chronic morphine-drinking rats treated with vehicle displayed a marked increase in the length and thickness of astrocytic processes (**** $p < 0.0001$, One-way ANOVA followed by Tukey's post-hoc test) and microglial density (red bars in **B–D**) (** $p < 0.01$, One-way ANOVA followed by Tukey's post-hoc test) compared with water drinking rats (green bars in **B–D**). The simultaneous intranasal and intravenous administration of three secretome doses (on day 22 of the chronic morphine access and on days 2 and 9 of the deprivation period) significantly reduced the length and thickness of primary astrocytic processes (blue bars in **B** and **C**) (**** $p < 0.0001$, One-way ANOVA followed by Tukey's post-hoc test), but not the microglial density (blue bar in **D**) compared with the morphine + vehicle treated animals (red bars in **B–D**). **(E)** GSSG/GSH ratio in the hippocampus of rats chronically drinking morphine, deprived for 12 days, and allowed morphine re-access for 24 hours. Chronic morphine drinking rats treated with vehicle showed a three-fold increase in the GSSG/GSH ratio (red bar) (* $p < 0.05$, One-way ANOVA followed by Tukey's post-hoc test) compared with rats drinking only water (green bar). The simultaneous intranasal and intravenous administration of three secretome doses (on day 22 of the chronic morphine access and on days 2 and 9 of the deprivation period) resulted in the full normalization of the GSSG/GSH ratio (blue bar) (* $p < 0.05$, One-way ANOVA followed by Tukey's post-hoc test). **F** MDA levels in the neostriatum. Chronic morphine drinking rats treated with vehicle showed a two-fold increase in MDA levels (red bar) (**** $p < 0.0001$, One-way ANOVA followed by Tukey's post-hoc test) compared with rats drinking only water (green bar). Simultaneous intranasal and intravenous administration of three secretome doses resulted in the near complete normalization of the MDA levels (blue bar) (** $p < 0.01$, One-way ANOVA followed by Tukey's post-hoc test). Data are presented as mean $\pm$ SEM; $n = 9$ for each experimental condition.

and 9 of the deprivation period) and allowed morphine (150 mg/l) re-access for 24 hours (water was always available through both the deprivation period and morphine re-access). We observed that upon morphine re-access, secretome-treated animals ingested a significantly lower amount of morphine ($p < 0.01$, Two-tailed Student t test) (Fig. 1C) and showed a reduced morphine preference ($p < 0.01$, Two-tailed Student t test) (Fig. 1D) compared with vehicle-treated animals.

Simultaneous intranasal and intravenous administration of secretome derived from human preconditioned MSCs to Wistar rats chronically ingesting morphine normalizes the increased astrocyte reactivity and brain oxidative stress induced by morphine intake. Glial cells are the central players in the induction and maintenance of brain inflammation [50]. As indicated, neuroinflammation and oxidative stress self-perpetuate each other and may be responsible for relapse drug intake despite prolonged drug abstinence [8]. Thus, neuroinflammation was evaluated determining glial reactivity, evidenced as morphological changes in astrocytes and microglial density in the hippocampus. To determine whether secretome administration reduced morphine-induced neuroinflammation animals were euthanized immediately after the 24-hour morphine re-access and their hippocampi were processed for astrocyte and microglia immunofluorescence. Animals that were never exposed to morphine (water group) were used as controls. As expected, chronic morphine intake induced a marked astrocytosis evidenced by a significant increase in the length ($p < 0.0001$, One-way ANOVA followed by Tukey's post-hoc test) (Fig. 2A top and B) and thickness ($p < 0.0001$, One-way ANOVA followed by Tukey's post-hoc test) (Fig. 2A top and C) of the primary astrocytic processes compared with those of water drinking animals. Secretome administration fully reversed the increase in the length and thickness of primary astrocytic processes ($p < 0.0001$, One-way ANOVA followed by Tukey's post-hoc test) (Fig. 2A top and B and C). Morphine ingesting animals also showed a significant increase in microglial density ($p < 0.01$, One-way ANOVA followed by Tukey's post-hoc test) (Fig. 2A center and D) compared with those of water drinking animals. Secretome administration had no significant impact on morphine-increased microglial density (Fig. 2A center and D).

Oxidative stress was determined as the ratio of oxidized/reduced glutathione (GSSG/GSH) in the hippocampus and by the level of lipid peroxidation in the neostriatum (two brain structures of the reward system) since both are highly sensitive hallmark indicators of the brain cellular redox state [51]. We observed that GSSG/GSH ratio in the hippocampus of rats chronically ingesting morphine, deprived of morphine for 12 days and allowed

morphine re-access for 24 hours was increased 3.5-fold compared to that in water drinking animals ($p < 0.05$, One-way ANOVA followed by Tukey's post-hoc test) (Fig. 2E). Three intranasal and intravenous administrations of MSC-derived secretome (one during the chronic morphine intake and two during the deprivation period) fully restored the normal GSSG/GSH ratio ($p < 0.05$, One-way ANOVA followed by Tukey's post-hoc test) (Fig. 2E). Similar results were observed for the evaluation of MDA levels in the neostriatum (Fig. 2F); thus, indicating that protracted neuroinflammation and oxidative stress induced by voluntary morphine intake were suppressed by secretome administration.

Simultaneous intranasal and intravenous administration of secretome derived from human preconditioned MSCs to Wistar rats chronically ingesting morphine does not modify the increase of morphine-induced expression of neither μ -opioid receptor nor glutamate transporters. The μ -opioid receptor 1 (OPRM1) is the main subtype receptor for morphine in the central nervous system [52], exhibiting a clear role in morphine-induced analgesia and morphine rewarding effects and dependence [53]. Changes in the activity of glial glutamate transporters, which regulate glutamate uptake have also been implicated in the development of morphine dependence [54]. Thus, we measured in prefrontal cortex and nucleus accumbens the mRNA levels of (a) OPRM1, (b) xCT and (c) GLT-1, 24 hour after morphine re-access. Morphine drinking animals showed a significant increase in mRNA levels of OPRM1 ($p < 0.05$, One-way ANOVA followed by Tukey's post-hoc test) in prefrontal cortex and nucleus accumbens (Fig. 3A) and, an unexpected increase in xCT mRNA level ($p < 0.05$, One-way ANOVA followed by Tukey's post-hoc test) in prefrontal cortex and nucleus accumbens (Fig. 3B) and in GLT-1 mRNA level ($p < 0.05$, One-way ANOVA followed by Tukey's post-hoc test) in prefrontal cortex only (Fig. 3C) compared to water drinking animals. Secretome administration to morphine dependent animals did not alter morphine-induced mRNA levels of OPRM1, xCT and GLT-1 since differences in the mRNA levels of these molecules were not significant compared to morphine dependent animals treated with the vehicle.

Model 2: Wistar-derived UChB rats

Intranasal administration of secretome derived from human preconditioned MSCs inhibits chronic morphine intake and reduces post-deprivation morphine re-access intake in UChB rats. Numerous studies have identified the contribution of heredity on drug dependence and the possible role of common pathways associated with dependence for multiple drugs [55]. The Wistar-derived UChB rat line, selectively bred during ninety generations

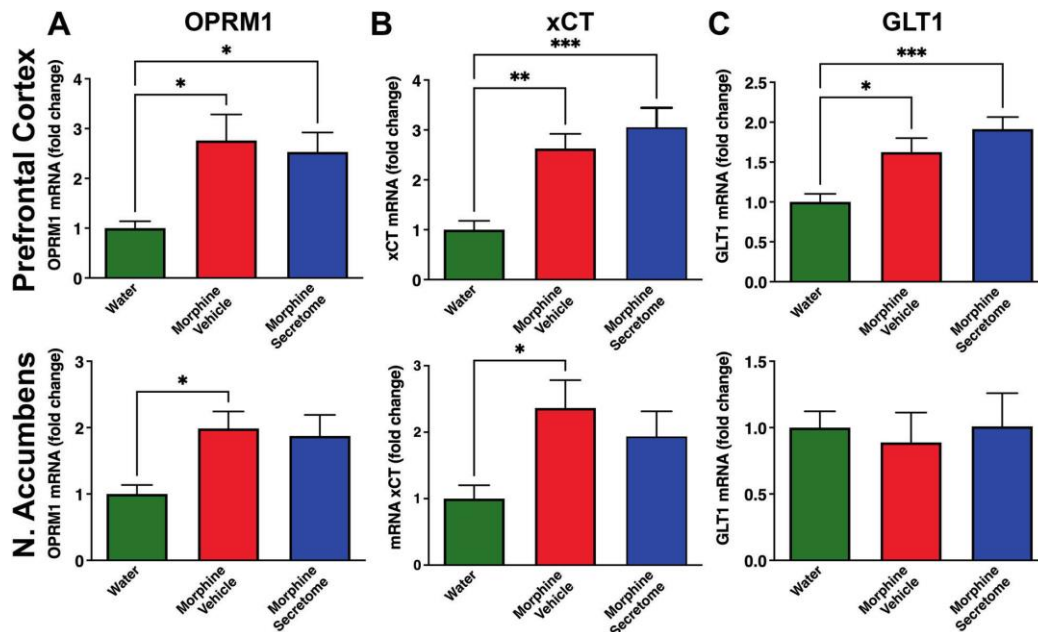


Fig. 3 Simultaneous intranasal and intravenous administration of secretome derived from human preconditioned MSCs to Wistar did not modify the morphine-induced increases in the brain expression of μ -opioid receptor or glutamate transporters. Quantification of the mRNA levels of the (A) μ -opioid receptor OPRM1; (B) xCT glutamate transporter and (C) GLT-1 glutamate transporter, determined by RT-qPCR in prefrontal cortex (top) and nucleus accumbens (bottom) of rats chronically drinking morphine, deprived for morphine for 12 days and allowed morphine re-access for 24 hours. Data were normalized against the mRNA level of the housekeeping gene GAPDH in the same sample. Morphine intake induced a significant increase in μ -opioid receptor mRNA levels in prefrontal cortex and nucleus accumbens (red bars in A) (* p < 0.05, One-way ANOVA followed by Tukey's post-hoc test), in xCT mRNA levels in prefrontal cortex and nucleus accumbens (red bars in B) (* p < 0.05, ** p < 0.01 One-way ANOVA followed by Tukey's post-hoc test), and in GLT-1 mRNA levels in prefrontal cortex (red bars in C) (* p < 0.05, One-way ANOVA followed by Tukey's post-hoc test), compared with water drinking animals (green bars). Simultaneous intranasal and intravenous administration of three secretome doses (on day 22 of the chronic morphine access and on days 2 and 9 of the deprivation period) had no impact on the mRNA levels of these molecules. Data are presented as mean $\pm$ SEM; n = 9 for each experimental condition.

for its high voluntary ethanol consumption [36, 37], appears as a useful model to study oral poly-drug addiction [34, 35]. Thus, we evaluated if secretome administration could reduce chronic morphine intake in the UChB rat model. UChB rats were pretreated with a daily intraperitoneal dose of morphine (40 mg/kg) for 9 days to promote subsequent voluntary morphine intake. After discontinuation of systemic morphine administration, the animals were offered free-choice access between two bottles, one containing water and the other a morphine solution of increasing concentrations on successive days; a paradigm in line with studies of nicotine self-administration [41, 42] (Supplementary Fig. 1B). A progressive escalation of voluntary oral morphine consumption was achieved by increasing the concentration of the morphine solution offered (Supplementary Fig. 3A). Following 15 days of access to 44 and 50 mg/l morphine solutions and water, animals achieved a stable oral morphine consumption of 7.4 ± 0.2 mg morphine/kg/day. After 10 weeks of continuous voluntary morphine consumption, animals were divided into two groups and treated with an intranasal dose of secretome (25 μ g protein) derived from 1×10^6 preconditioned MSCs or vehicle. It has been previously reported that, by this route of administration, MSC-secretome is effective in reducing voluntary alcohol and nicotine intake in UChB rats [34]. As shown in Fig. 4A-Left, the first intranasal secretome administration induced a 37% reduction (p < 0.0001, Two-way ANOVA) in voluntary morphine intake compared to that of vehicle-treated animals. The intranasal administration of additional secretome doses given every five

days for two weeks significantly enhanced (p < 0.0001, Two-tailed Student t test) the inhibitory effect of secretome, reaching a 72% reduction in morphine intake after three intranasal secretome doses (2.2 ± 0.4 mg morphine/kg/day for secretome-treated animals versus 7.7 ± 0.2 mg morphine/kg/day for vehicle-treated animals). The half-life of the secretome inhibitory action on morphine intake is of the order of 6–7 days, such that the secretome administration every 5 days results in a cumulative effect. As expected, the reduction in morphine intake induced by secretome administration was associated with a significant reduction (p < 0.01 to 0.0001, Two-way ANOVA) in morphine preference versus water (Fig. 4B-Left). Secretome-induced inhibition of morphine intake was counterbalanced by increases in water intake (p < 0.0001, Two-way ANOVA) (Fig. 4C Left), thus indicating that rats kept their hydric homeostasis. Four days after the last intranasal dose of secretome, animals were deprived of the morphine solutions for five days and treated with a fourth dose of secretome or vehicle during the first day of deprivation. Subsequently, rats were allowed re-access to the 44- and 50 mg/l morphine solutions for one day to evaluate post-deprivation relapse intake. Animals in the vehicle-treated group ingested 7.2 ± 0.2 mg morphine/kg/day while animals in the secretome-treated group ingested 2.0 ± 0.2 mg morphine/kg/day; thus, a 72% reduction (p < 0.0001, Two-tailed Student t test) in post-deprivation morphine relapse intake (Fig. 4A-Right). Secretome administration also significantly reduced (p < 0.0001, Two-tailed Student t test) morphine preference over water upon re-access

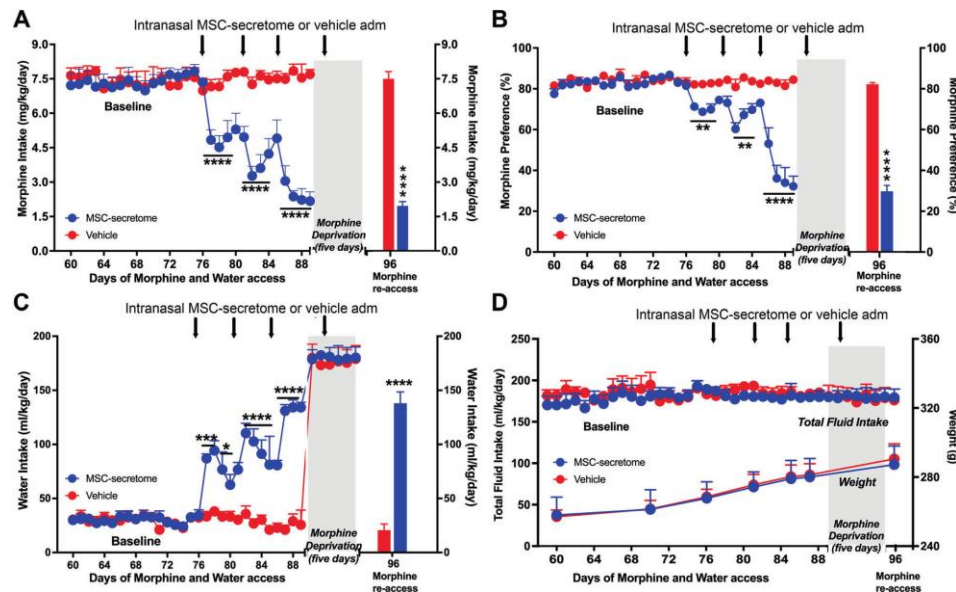


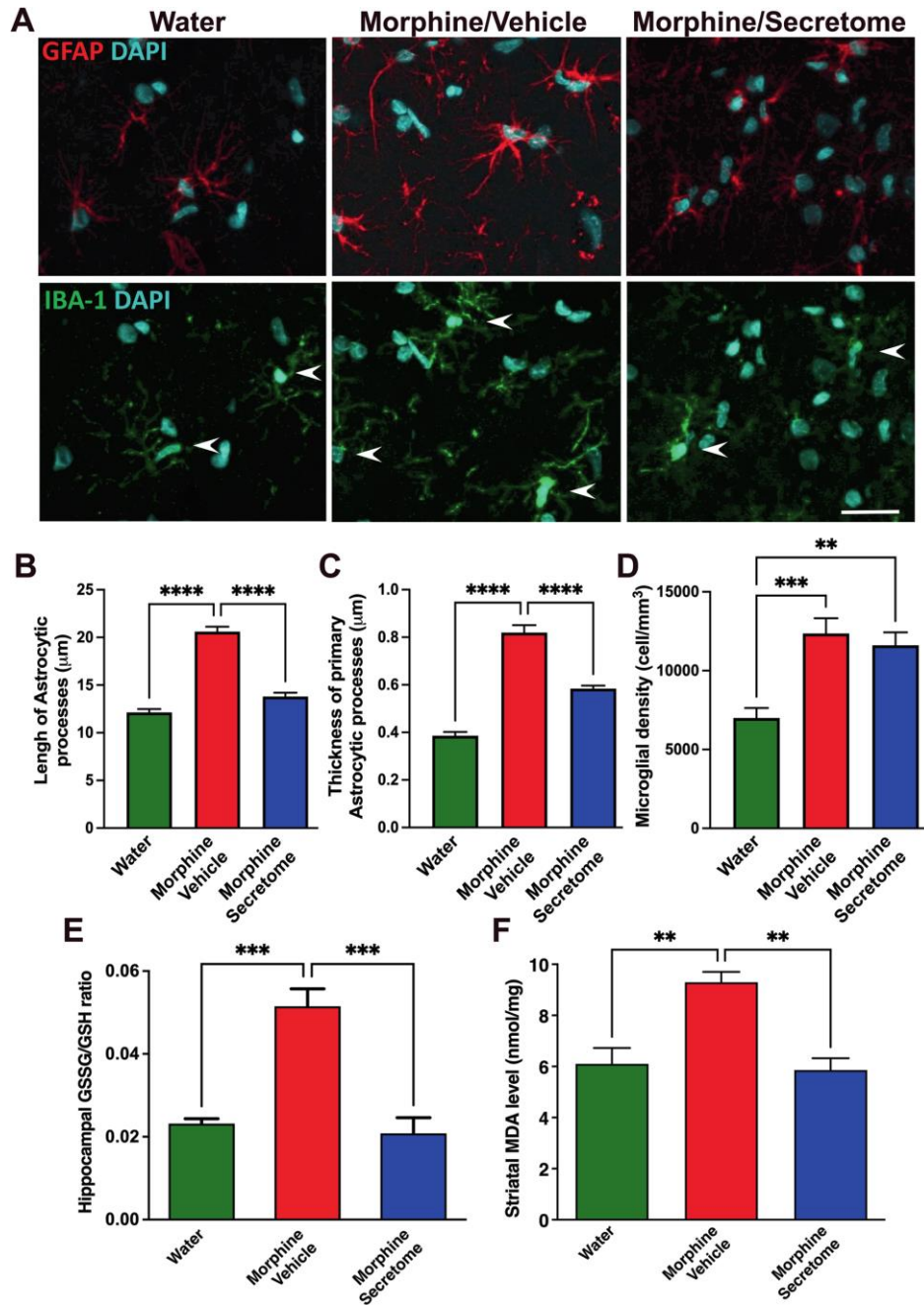
Fig. 4 Repeated intranasal administration of secretome derived from human preconditioned MSCs to UChB rats that had chronically consumed morphine inhibits its intake and reduces post-deprivation relapse. **A-Left** Daily morphine intake and **(B-Left)** morphine preference over water. UChB rats that had consumed morphine for 76 days were intranasally treated with three doses of secretome derived from preconditioned MSCs or vehicle (indicated by arrows). Two-way ANOVA (treatment $\times$ day) of morphine intake following three intranasal doses of MSC secretome indicates significant effect of treatment [$F_{(1,180)} = 195.4, p < 0.0001$], day [$F_{(29,180)} = 8.76, p < 0.0001$], and significant interaction [$F_{(29,180)} = 10.15, p < 0.001$] compared with control rats receiving vehicle. Tukey's post-hoc analysis revealed that secretome treatment (blue circles) inhibited morphine intake during the 14 days recorded period, versus vehicle-treated control (red circles) ($***p < 0.001$). The inhibition of morphine intake induced by the third secretome dose (72%) was significantly higher ($**p < 0.01$) than the inhibition induced by the second dose (50%) and the inhibition induced by the latter was significantly higher ($*p < 0.05$) than the inhibition induced by the first dose (34%) (two-tailed Student *t* test). **(A Right)** Morphine intake and **(B Right)** morphine preference over water of UChB rats after five days of morphine deprivation followed by one day of free choice of 44 mg/l and 50 mg/l morphine re-access. Rats treated previously with four intranasal secretome doses (blue bar) ingested a 74% lower amount of morphine than vehicle treated animals (red bar) ($****p < 0.0001$; Two-tailed Student *t* test). **(C Left)** Daily water intake showing that intranasal administration of three secretome doses (blue circles) significantly increased water intake compared with vehicle treated rats (red circles). Two-way ANOVA (treatment $\times$ day) indicates significant effect of treatment [$F_{(1,216)} = 213.9, p < 0.0001$], day [$F_{(35,216)} = 104.7, p < 0.0001$], and significant interaction [$F_{(35,216)} = 11.92, p < 0.0001$]. Tukey's post-hoc analysis revealed that secretome treatment increases water intake during the 14 days recorded versus vehicle-treated control ($***p < 0.001$). The higher water intake induced by the third secretome dose (80%) was significantly higher than the increase induced by the first dose (57%) ($*p < 0.05$, two-tailed Student *t* test). **(C Right)** Water intake after five days of morphine deprivation followed by one day of 44 mg/l and 50 mg/l morphine solutions re-access. Rats treated previously with four intranasal secretome doses (blue bar) ingested a significantly higher amount of water than vehicle control animals (red bar) ($****p < 0.0001$, Two-tailed Student *t* test). **(D)** Total fluid intake and animals body weights of UChB rats intranasally treated with secretome derived from preconditioned MSCs (blue circles) or vehicle (red circles). Data showed that body weight or total fluid intake were not affected by secretome administration, indicating that effects induced by this treatment were specific for morphine intake. Data are presented as mean $\pm$ SEM; $n = 4$ for each experimental condition.

(Fig. 4B-Right). As also observed in the Wistar rat model, secretome administrations had no impact on total fluid intake or animal weight in UChB rats (Fig. 4D).

Intranasal administration of secretome derived from human preconditioned MSCs reduces morphine-induced astrocyte activation and brain oxidative stress in UChB rats. As for the Wistar rat model, we evaluated whether chronic morphine intake also induced neuroinflammation and brain oxidative stress in UChB rats and whether these parameters could be normalized by secretome administration. To this end, animals were euthanized after the 24-hour morphine re-access. Animals that were never exposed to morphine (water group) were used as controls. Once again, chronic morphine intake induced a marked astrocytosis evidenced by a significant increase in the length ($p < 0.0001$, One-way ANOVA followed by Tukey's post-hoc test) (Fig. 5A-top and Fig. 5B) and thickness ($p < 0.0001$, One-way ANOVA followed by Tukey's post-hoc test) (Fig. 5A-top and C) of primary astrocytic

processes in hippocampus compared with those in water drinking animals. Secretome administration fully reversed the increase in the length and thickness of primary astrocytic processes ($p < 0.0001$, One-way ANOVA followed by Tukey's post-hoc test) (Fig. 5A-top, B and C). Morphine ingesting animals also showed a significant increase in microglial density ($p < 0.001$, One-way ANOVA followed by Tukey's post-hoc test) (Fig. 5A-center and D) compared with that in water drinking animals, while the administration of secretome had no impact on morphine-increased microglial density (Fig. 5A-center and D).

As also seen in Wistar rats, UChB rats showed a significant increase ($p < 0.001$, One-way ANOVA followed by Tukey's post-hoc test) of GSSG/GSH ratio in hippocampus (Fig. 5E) and of MDA levels ($p < 0.01$, One-way ANOVA followed by Tukey's post-hoc test) in neostriatum (Fig. 5F) of morphine-drinking compared to water drinking animals. Intranasal secretome administration fully restored the GSSG/GSH ratio ($p < 0.001$, One-way ANOVA followed by Tukey's post-hoc test) (Fig. 5E) and the MDA levels ($p < 0.01$,



One-way ANOVA followed by Tukey's post-hoc test) to levels observed in animals that were not exposed to morphine (Fig. 5F).

Single intranasal administration of secretome derived from human preconditioned MSCs during the morphine deprivation period

reduces morphine relapse in UChB rats. Subsequent studies evaluated whether the administration of a single intranasal dose of secretome derived from preconditioned MSCs, given only during the deprivation period, reduced the post-deprivation morphine relapse upon morphine re-access. To increase voluntary

Fig. 5 Intranasal administration of secretome derived from human preconditioned MSCs reduces morphine-induced astrocyte activation and brain oxidative stress in UChB rats. **A** Representative confocal microphotographs of GFAP immunoreactivity (red top) for hippocampal astrocytes and Iba-1 immunoreactivity (green, shown by arrows, center) for hippocampal microglia. Nuclei were counterstained with DAPI (blue, nuclear marker), scale bar 25 μ m. **B** Quantification of length and **(C)** thickness of primary astrocytic processes. **D** Quantification of microglia density. Chronic morphine-ingesting rats treated with vehicle displayed a marked increase in the length and thickness of astrocytic processes (**** p < 0.0001, One-way ANOVA followed by Tukey's post-hoc test) and microglial density (red bars in **B–D**) (*** p < 0.001, One-way ANOVA followed by Tukey's post-hoc test) compared with water drinking rats (green bars in **B–D**). The intranasal administration of secretome significantly reduced the length and thickness of primary astrocytic processes (blue bars in **B** and **C**) (**** p < 0.0001, One-way ANOVA followed by Tukey's post-hoc test) compared with the morphine vehicle treated animals (red bars in **B** and **C**). **E** Quantification of GSSG/GSH ratio in the hippocampus. Chronic morphine drinking rats treated with vehicle showed a 2.5-fold increase in GSSG/GSH ratio (red bar) (*** p < 0.001, One-way ANOVA followed by Tukey's post-hoc test) compared with rats drinking only water (green bar). Intranasal administration of secretome resulted in the full normalization of the GSSG/GSH ratio (blue bar) (*** p < 0.001, One-way ANOVA followed by Tukey's post-hoc test). **F** Quantification of MDA levels in neostriatum. Chronic morphine drinking rats treated with vehicle showed a 60% increase in MDA levels (red bar) (** p < 0.01, One-way ANOVA followed by Tukey's post-hoc test) compared with rats drinking only water (green bar). Intranasal secretome administration resulted in the full normalization of MDA levels (blue bar) (** p < 0.01, One-way ANOVA followed by Tukey's post-hoc test). Data are presented as mean $\pm$ SEM; n = 4 for each experimental condition.

morphine intake and to induce a more potent morphine relapse, the previous paradigm of morphine self-administration was slightly modified. The priming schedule of daily intraperitoneal administration of 40 mg/kg morphine was extended from 9 to 11 days and the morphine concentrations offered on the subsequent two-bottle choice was increased at a faster pace, reaching 90 mg/l within 41 days of morphine exposure (Supplementary Fig. 1C). Rats subjected to this paradigm consumed 14.1 ± 0.1 mg of morphine/kg/day (Supplementary Fig. 3B).

After eight weeks of continuous voluntary oral morphine consumption, animals were deprived of morphine solutions for six days. Following four days of deprivation rats were administered a single intranasal dose of secretome (25 μ g protein) derived from 1×10^6 preconditioned MSCs or vehicle while the deprivation was continued for two additional days, after which animals were allowed re-access to the 80 and 90 mg/l morphine solutions for two days (water was always available through all the experiment). The single-dose secretome administration induced a 85% reduction of morphine relapse intake (p < 0.0001, Two-tailed Student t test) assessed 24 hours after morphine re-access, compared to vehicle-treated rats (2.9 ± 0.6 mg morphine/kg/day for secretome-treated animals versus 14.3 ± 0.5 mg morphine/kg/day for vehicle-treated animals), an effect that was maintained in the second re-access day (Fig. 6A). Secretome administration during the deprivation period also reduced morphine preference over water upon re-access (Fig. 6B) and increased water intake (Fig. 6C), without affecting total fluid intake or animal weight (Fig. 6D).

DISCUSSION

Studies conducted on a model of opioid dependence in Wistar rats which reproduce a severe naloxone-induced withdrawal [10], demonstrated that secretome derived from preconditioned human mesenchymal stem cells administered intranasally and systemically (i) virtually abolished morphine dependence as shown by a > 95% reduction of voluntary morphine consumption, (ii) fully suppressed morphine-induced brain oxidative stress determined by the normalization of increases in GSSG/GSH ratio in the hippocampus and lipid peroxidation in the neostriatum, and (iii) inhibited neuroinflammation evidenced by a full reversal of astrocytic morphological changes induced by chronic morphine self-administration.

The second model of voluntary oral morphine intake was implemented in rats bred as heavy alcohol drinkers (UChB), since these animals have shown a strong drug-permissive genetic background. In such a model, rats orally self-administered morphine solutions of increasing concentrations after systemic morphine administration. In this animal model, intranasal administration of a secretome dose once every five days for 15 days (i) inhibited voluntary morphine intake by 72%, (ii) normalized the

GSSG/GSH ratio and the lipid peroxidation index and (iii) reduced neuroinflammation evidenced as a full reversal of astrocyte morphological changes induced by chronic morphine self-administration in the hippocampus, an area related to memory consolidation.

The inhibition of morphine intake reported in both models is consistent with the finding that secretome antagonized both the oxidative stress and the neuroinflammation which characterize the use of addictive drugs [8–10]. Indeed, morphine consumption induces oxidative stress and elevates NO synthesis following μ -opioid receptor activation [8, 11], while neuroinflammation results from the use of opioids [12], mainly activating NF κ B signaling leading to the production of pro-inflammatory mediators [10, 13, 14]. The results in the present study are in line with previous reports in animal models of opioid consumption showing that systemic administration of ibuprofen, an anti-inflammatory molecule that reduces TNF- α levels, reduces opioid withdrawal [56], while intraperitoneal administration of the antioxidant N-acetylcysteine, which improves glutamate uptake, also reduced relapse [57]. In the same line, Motaghinejad et al. reported that the daily intraperitoneal administration of the polyphenol curcumin, with recognized antioxidant and anti-inflammatory properties, lowered the increased levels of brain oxidized glutathione and lipid peroxidation in morphine-treated rats [58], attenuating morphine tolerance and dependence. Thus, the above studies strengthen the idea that blocking the inflammatory and pro-oxidative effects of morphine may provide a promising therapeutic strategy.

Brain oxidative stress and neuroinflammation following the use of addictive drugs [8, 10, 59]; interact in a long-acting vicious cycle which self-perpetuate each other and inhibit the tripartite synaptic glutamate removal by the astrocyte glutamate transporter GLT-1. Indeed, a hyperglutamatergic tonus has been reported for all addictive drugs [60]; in line with the finding that reinstatement of heroin seeking by conditioned cues is associated with glutamate overflow in the nucleus accumbens [61], and with the observation that glutamate and malondialdehyde (MDA) levels and GSSG/GSH ratio were progressively increased in the brain of morphine treated mice [62, 63]. The potent anti-inflammatory and antioxidant effects of MSC-derived secretome are well documented in animal models of stroke [64], perinatal asphyxia [65] and traumatic brain injury [66], where behavioral dysfunctions and markers of neuroinflammation and oxidative stress are reduced after secretome administration.

It is noted that MSC administration has been tested for the treatment of *opioid tolerance*, one of the complications commonly seen after chronic opioid medical use, which is also associated with neuroinflammation, but in the spinal cord [67]. This report indicated that the intravenous administration of rat MSCs to animals that had received daily morphine injections significantly

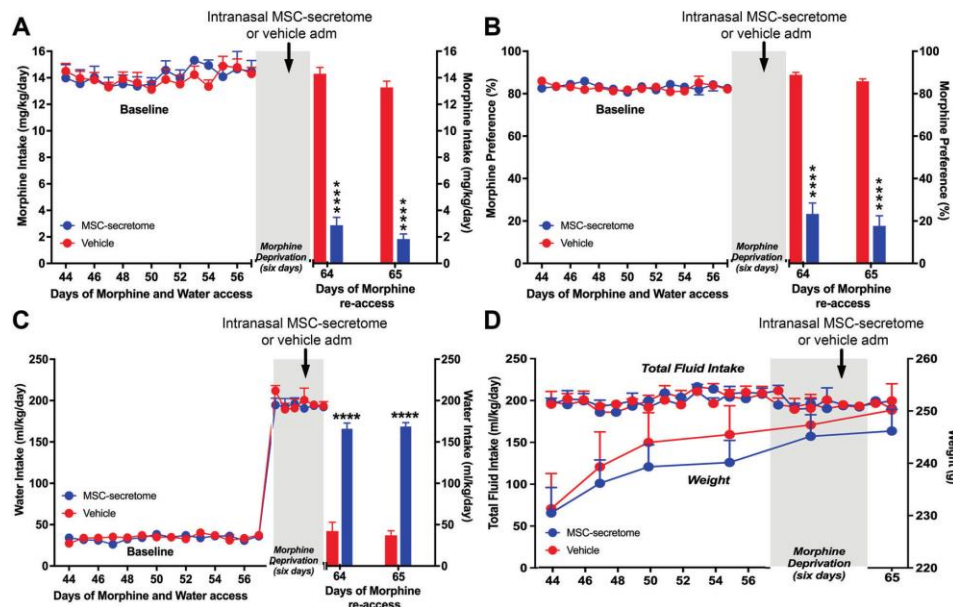


Fig. 6 A single intranasal administration of secretome derived from human preconditioned MSCs during the deprivation period to UChB rats that had chronically consumed morphine markedly reduces post-deprivation relapse. **A** Morphine relapse intake and **(B)** Morphine preference over water of UChB rats that had voluntarily consumed morphine for 57 days were morphine deprived for six days and intranasally treated with a single dose of secretome derived from preconditioned MSCs or vehicle (indicated by the arrow) on day 61. Relapse morphine intake and morphine preference upon re-access to the 80 and 90 mg/l morphine solutions over water was significantly reduced in secretome treated rats (80% to 85% **** $p < 0.0001$, two-tailed Student *t* test) on the two re-access days (blue bars in **A** and **B**) compared with vehicle treated animals (red bars in **A** and **B**). **C** Daily water intake showing that secretome administration during the morphine deprivation period significantly increased water intake (days 64 and 65) (**** $p < 0.0001$, Two-tailed Student *t* test) upon morphine re-access (blue bar) compared with vehicle-treated animals (red bar). **D** Total fluid intake and animals body weight of UChB rats intranasally treated with secretome derived from preconditioned MSCs (blue circles) or vehicle (red circles) during the deprivation period. Data shows that these parameters were not affected by secretome administration. Data are presented as mean $\pm$ SEM; $n = 6$ for each experimental condition.

reduced opioid tolerance, restoring the sensitivity to morphine [67]. In line with our results, the reported reduction of morphine tolerance was related to the ability of MSCs to reduce inflammation, decreasing the activation of astrocytes in the spinal cord [67]. Previously, in animal models of ethanol and nicotine dependence, we reported that the intranasal administration of MSC-derived secretome was able to reduce both astrocytic activation and microglial density [34], while for opioid dependence astrocytes appear as the main players in the regulation of opioid relapse.

In the present studies, molecular markers of opioid-induced dependence were examined in prefrontal cortex and nucleus accumbens since these brain areas are highly involved in morphine addiction [68]. As previously reported, we observed that chronic morphine intake induced a significant increase in OPRM1 mRNA levels, the main morphine receptor, in prefrontal cortex and nucleus accumbens [10], but also an unexpected increase in mRNA levels of the glutamate transporters GLT-1 and xCT. The reason for the increase in mRNA levels of glutamate transporters by chronic morphine intake is not known. Nevertheless, these could be associated with a compensatory mechanism related to an increased oxidation of glutamate transporters induced by ROS generated by the chronic morphine consumption, since these transporters present complex regulations [69]. Secretome administration had no impact on morphine-induced mRNA levels of these molecules.

While treatment of opioid-dependent patients without prior waiting for a protracted opioid withdrawal, as shown in the models presented here appears promising, abstinent addicts are

at high risk of relapse due to conditioned craving elicited by drug-paired cues [70], leading to a relapse rate of more than 90% after the first month of abstinence [71]. Thus, we also evaluated whether secretome administration given only during the deprivation period could also reduce opioid intake upon re-access. We observed that a single intranasal administration of secretome during the morphine withdrawal period markedly reduced (85%) relapse opioid intake upon re-access, suggesting that MSC-derived secretome could be a useful biobdrug for the treatment of opioid addiction.

It is noted that in the Wistar rat (model 1) study, aiming at blocking both the peripheral [72] and central effects of morphine [73], MSCs secretome was administered a single time both intravenously and intranasally. This dual route fully abolished (>95%) morphine dependence intake, an effect observed mostly within the first 48 hours. Such a finding is noteworthy given that in this animal model, naloxone administration generates a most pronounced withdrawal reaction [10]. The UChB rat (model 2) studies showed that by itself the intranasal route was only partially effective in reducing self-administration while a single dose was most effective in reducing post-deprivation relapse (80–85%). The intranasal route may be valuable for the withdrawn opioid user for self-application after a methadone tapering period.

Further studies are required to determine the full duration of the anti-relapse action of secretome and the number of doses needed to maintain the observed therapeutic effects. However, since intranasal administration of MSC-derived secretome has also shown to have potent therapeutic-like effects in animal models of

alcohol and nicotine addiction [34], data suggest that this biodrug could have therapeutic potential for polydrug users; a frequent situation [74] for which there is no FDA approved medication [75].

It is noted that opioid substitution has become the treatment of choice for heroin dependence, and methadone and buprenorphine are the substitution drugs most often prescribed. Importantly, polydrug use is particularly prevalent among this group of patients (EMCDDA 2009). In the Netherlands, one half of the methadone users reported cocaine as their secondary drug (IVZ, 2004). Cocaine use can also increase alcohol use, and about 60% of patients in a methadone maintenance program who also used crack cocaine reported using alcohol to come down [76]. In Barcelona, more than one-third of patients were found to be polydrug users when they began methadone maintenance treatment [77]. In essence, polydrug users become opioid dependent, thus greatly adding to the number users at risk of a fentanyl overdose.

A question that can be asked is whether the opioid dependent patient receiving secretome will change to another drug. It is known that the use of several substances by an individual reflects the replacement of one drug by another, due to changes in price, availability, legality, or fashion [78]. Thus, it can also be suggested that drug changes may also occur if there is a secretome-reduced pharmacological potency of the opioid. However, several studies suggest a common mechanism of relapse for different drugs of abuse [17]. Such a view might explain our reports that mesenchymal stem cells and/or its secreted products inhibit drug relapse of several drug types including ethanol, nicotine and now opioids [30, 31, 34, 79, 80].

If the findings in present studies are successfully reproduced in the clinical situation, the administration of mesenchymal stem cell secretome to opioid dependent patients could also have a major economic impact. The Pew-Trust in 2021 [81] estimated the cost of the opioid epidemic in the United States to be \$131.8 billion dollars (Health care cost \$35 billion; Criminal Justice 14.8 billion; Loss of productivity due to premature death due to overdose and productive hours due to AUD and incarceration: \$92 billion).

Overall, the studies conducted show that the administration of secretome derived from human preconditioned MSCs abolishes the opioid-induced neuroinflammation and brain oxidative stress, leading to a significant reduction in chronic opioid intake and opioid relapse. The reported preclinical data may have translational value in reducing the opioid epidemic.

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ACKNOWLEDGEMENTS

This work was supported by FONDECYT 1200287 grant to Fernando Ezquer, ACT210012 grant to Fernando Ezquer and Yedy Israel and FONDECYT 3210276 grant to Pablo Berrios-Cárcamo. The technical assistance of Mrs. Camila Ezquer, Mr. Jorge Ruiz, Ms. Catalina Vallejos, Mr. Robel Vasquez, Mr. Juan Santibañez and Ms. Carmen Almeyda is greatly appreciated.

AUTHOR CONTRIBUTIONS

MEQ*: Conception and design, collection of data, data analysis, final approval of manuscript; MQ*: Conception and design, collection of data, data analysis, final approval of manuscript; PM: Collection of data, data analysis, final approval of manuscript; PBC: Collection of data, data analysis, final approval of manuscript; DS: Collection of data, data analysis, final approval of manuscript; ME: Data analysis, financial support, final approval of manuscript; MHM: Data analysis, final approval of manuscript; YI: Conception and design, financial support, data analysis, manuscript writing, final approval of manuscript. FE*: Conception and design, financial support, collection of data, data analysis, manuscript writing, final approval of manuscript. *Authors who contributed equally to this work.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41398-022-02225-0>.

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6. Chapter 3

Amelioration of morphine withdrawal syndrome by systemic and intranasal administration of mesenchymal stem cell-derived secretome in preclinical models of morphine dependence

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ID: CNSNT-2023-1115 (REX-PROD-1-1477F9CA-68E6-4CC5-B95E-73F49F985126-A7C190F2-A4D9-4A37-8DB3-0C96591E460B-88357)

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Journal:	<i>CNS Neuroscience & Therapeutics</i>
Manuscript ID	CNSNT-2023-1115
Wiley - Manuscript type:	Original Article
Date Submitted by the Author:	31-Jul-2023
Complete List of Authors:	Ezquer, Fernando; Universidad del Desarrollo Facultad de Medicina; Agencia Nacional de Investigacion y Desarrollo Quezada, Mauricio; Universidad del Desarrollo Facultad de Medicina Ponce, Carolina; Universidad de Chile Facultad de Medicina Berríos-Cárcamo, Pablo; Universidad del Desarrollo Facultad de Medicina Santapau, Daniela; Universidad del Desarrollo Facultad de Medicina Gallardo, Javiera; Universidad del Desarrollo Facultad de Medicina Gregorio, Cristian De; Universidad del Desarrollo Facultad de Medicina Quintanilla, María Elena; Universidad de Chile Facultad de Medicina Morales, Paola; Universidad de Chile Facultad de Medicina Ezquer, Marcelo; Universidad del Desarrollo Facultad de Medicina Herrera-Marschitz, Mario; Universidad de Chile Facultad de Medicina Israel, Yedy; Universidad de Chile Facultad de Medicina Andrés-Herrera, Paula; Universitat de Valencia Departament de Farmacia i Tecnologia Farmaceutica i Parasitologia; Universitat de Valencia Facultat de Farmacia Hipólito, Lucia; Universitat de Valencia Departament de Farmacia i Tecnologia Farmaceutica i Parasitologia; Universitat de Valencia Facultat de Farmacia
Keywords:	Mesenchymal stem cells, opiod addiction, withdrawal, secretome, neuroinflammation
Scope of Manuscript:	Drug addiction, Neuroinflammation, Behavioural neurology, Astrocytes, Hippocampus, Mesenchymal stem cell

Amelioration of morphine withdrawal syndrome by systemic and intranasal administration of mesenchymal stem cell-derived secretome in preclinical models of morphine dependence

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Running Title: Stem cell secretome reduces opioid withdrawal

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29 ABSTRACT

30 **Background:** Morphine is an opiate commonly used in the treatment of moderate to severe pain.
31 However, prolonged administration can lead to physical dependence and strong withdrawal symptoms
32 upon cessation of morphine use. These symptoms can include anxiety, irritability, increased heart rate,
33 and muscle cramps, which strongly promote morphine use relapse. The morphine-induced increases
34 of neuroinflammation, brain oxidative stress, and alteration of glutamate levels in the hippocampus
35 and nucleus accumbens have been associated with morphine dependence and a higher severity of
36 withdrawal symptoms. Due to its rich content in potent anti-inflammatory and antioxidant factors,
37 secretome derived from human mesenchymal stem cells (hMSCs) is proposed as a preclinical
38 therapeutic tool for the treatment of this complex neurological condition associated with
39 neuroinflammation and brain oxidative stress.

40 **Methods:** Two animal models of morphine dependence were used to evaluate the therapeutic efficacy
41 of hMSC-derived secretome in reducing morphine withdrawal signs. In the first model, rats were
42 implanted subcutaneously with mini-pumps which released morphine at a concentration of 10
43 mg/kg/day for seven days. Three days after pump implantation, animals were treated with a
44 simultaneous intravenous and intranasal administration of hMSC-derived secretome or vehicle, and
45 withdrawal signs were precipitated on day seven by i.p. naloxone administration. In this model, brain
46 alterations associated with withdrawal were also analyzed before withdrawal precipitation. In the
47 second animal model, rats voluntarily consuming morphine for three weeks were intravenously and
48 intranasally treated with hMSC-derived secretome or vehicle, and withdrawal signs were induced by
49 morphine deprivation.

50 **Results:** In both animal models secretome administration induced a significant reduction of withdrawal
51 signs, as shown by a reduction in a combined withdrawal score. Secretome administration also
52 promoted a reduction in morphine-induced neuroinflammation in the hippocampus and nucleus
53 accumbens, while no changes were observed in extracellular glutamate levels in nucleus accumbens.

54 **Conclusion:** Data presented from two animal models of morphine dependence suggest that
55 administration of secretome derived from hMSCs reduces the development of opioid withdrawal signs,
56 which correlates with a reduction in neuroinflammation in the hippocampus and nucleus accumbens.

58 INTRODUCTION

59 The opioid dependence crisis has emerged as a significant global health concern (1), affecting millions
60 of individuals, negatively impacting their physical, mental, and social well-being. This situation has
61 been exacerbated during the COVID-19 pandemic, with the CDC's National Center for Health Statistics
62 reporting an increase of nearly 15% in opioid overdose deaths in the United States from 2020 to 2021
63 (2), with opioids responsible for almost 70% of all drug overdose deaths (3, 4). Morphine, a potent
64 opiate analgesic, is frequently used for severe pain management but also has a high potential for abuse
65 and dependence (5). One of the significant challenges faced by individuals attempting to discontinue
66 morphine use is the withdrawal syndrome, a set of severe physiological and psychological symptoms
67 experienced upon cessation or reduction of drug consumption (6). Withdrawal symptoms can vary in
68 intensity depending on the duration and type of opioid abuse, but also by individual factors (7, 8), often
69 leading to significant distress and discomfort. Consequently, a significant number of opioid-dependent
70 individuals return to opioid abuse to alleviate their symptoms (9), perpetuating the cycle of addiction
71 and dependence (10).

72
73 The main cause of withdrawal is related to the brain adaptation occurring while the individual is
74 subjected to a constant opioid stimulus and subsequent μ -opioid receptor activation (11), which is the
75 main target of most commonly abused opioids, including morphine (12). Specifically, μ -opioid receptor
76 activation results in reduction of cAMP levels and increase in potassium channels activation (11). These
77 effects reduce neuron excitability and affect brain areas with abundant μ -opioid receptor expression,
78 including areas related to affective responses like the hippocampus and the nucleus accumbens (13,
79 14). It is discussed whether the brain responds to this increased opioid activation by increasing its
80 excitatory tone, including increased glutamatergic activity (11, 15).

81
82 Recent evidence suggests that a deregulated tone in glutamatergic activity is increased by glial immune
83 activation. Opioids, including morphine, have been associated with an increase in both systemic and
84 brain oxidative stress and inflammation (16-19). Morphine can promote glial activation, and the

85 subsequent increase in oxidative stress and inflammatory markers, by the direct activation of μ -opioid
86 receptors (20, 21). In addition, morphine activates a sensor of foreign molecules, the toll-like receptor
87 4 (TLR4), promoting a pro-inflammatory signaling cascade and upregulation of pro-inflammatory
88 cytokines (22). The resulting neuroinflammation has been shown to be persistent, promoting the
89 increase of brain oxidative stress (20, 23, 24). An increase of neuroinflammation can alter behavior by
90 modulating glutamatergic neurotransmission, first by upregulation of oxidant enzymes, and then by
91 inactivation and downregulation of main glutamate transporters, like glutamate transporter-1 (GLT-
92 1), in charge of glutamate uptake, and possibly the glutamate interchanger system X_c^- and its catalytic
93 subunit xCT, that promotes the release of extrasynaptic glutamate (24-27). A reduction of these
94 transporters levels and activity have been correlated to increased spillover of glutamate in the nucleus
95 accumbens, increasing the activation of extrasynaptic glutamate receptors, in a model of heroin self-
96 administration and withdrawal (29). Consequently, an increase in GLT-1 levels in the hippocampus and
97 nucleus accumbens is correlated with reduced morphine and heroin relapse and withdrawal (23, 28,
98 29).

99
100 Conventional treatments for opioid withdrawal syndrome include opioid maintenance therapy, in
101 which long-acting opioids like methadone are prescribed to replace short-acting abused opioids like
102 morphine or fentanyl. However, this therapy has limited efficacy since patients often return to taking
103 the abused opioid when experiencing abstinence, and it does not address the brain adaptations that
104 trigger withdrawal symptoms and promote relapse (16, 30, 31). Thus, there is an urgent need to
105 explore novel therapeutic strategies to alleviate withdrawal symptoms, handling opioid abstinence.

106
107 One promising approach for treating complex neurological diseases associated to neuroinflammation
108 and brain oxidative stress is the use of secretome derived from human mesenchymal stem cells
109 (hMSCs) (23, 32-36). Secretome derived from hMSCs is a complex mixture of bioactive molecules,
110 containing proteins, lipids, and regulatory microRNAs (miRNAs), with potent anti-inflammatory,
111 immunomodulatory, and regenerative properties, that can be easily obtained by the *in vitro* culture of

112 these cells (23, 37). Given the prominent role of oxidative stress and neuroinflammation in opioid
113 withdrawal, the hMSCs secretome may offer a valuable therapeutic avenue for the management of
114 morphine withdrawal syndrome.

115
116 In this preclinical study, we aimed at evaluating the therapeutic effects of the simultaneous
117 intravenous and intranasal administration of hMSC-derived secretome on morphine withdrawal, using
118 two animal models of morphine dependence. This dual-route approach for secretome administration
119 was designed to maximize the therapeutic impact against opioid withdrawal, capitalizing on the unique
120 benefits of each route. The intranasal method provides a non-invasive delivery directly to the brain,
121 leveraging the olfactory and trigeminal pathways to bypass the blood-brain barrier. In contrast, the
122 intravenous route ensures a systemic distribution of the therapy, effectively addressing the
123 widespread symptoms of opioid withdrawal. This combined approach, thus, targets both the central
124 nervous system and systemic manifestations of opioid withdrawal.

125 In the first animal model, rats were implanted subcutaneously with osmotic mini-pumps (Alzet) that
126 infused morphine at a constant concentration of 10 mg/kg/day for seven days, and a withdrawal
127 syndrome was precipitated by administration of the μ -opioid antagonist naloxone three days after
128 secretome administration. In this model, in an additional group of animals, we also measured oxidative
129 stress and neuroinflammatory markers in the hippocampus and nucleus accumbens, and glutamate
130 extracellular levels in the nucleus accumbens, to assess the impact of hMSC secretome before the
131 withdrawal trigger. In the second animal model, rats voluntarily consumed morphine for 21 days,
132 reaching an oral morphine consumption of 17 mg/kg/day. In this model, the withdrawal syndrome was
133 spontaneously induced by morphine removal imposed immediately after secretome administration
134 and evaluated 48 hours later.

135
136 We hypothesized that secretome administration will decrease withdrawal behaviors and signs,
137 evaluated in both preclinical models of morphine dependence, in addition to a reduction in brain
138 oxidative stress and neuroinflammation, as well as normalization of glutamate levels.

139 MATERIALS AND METHODS

140

141 Animals

142 ***Animal model 1. Subcutaneous morphine administration and withdrawal syndrome precipitation by***

143 ***naloxone:*** 8-week-old female Wistar rats, weighing 180 to 220 g, were housed individually under
144 controlled environmental conditions, including a 12-hour light/dark cycle and at a constant room
145 temperature. They were under unrestricted access to rodent food and water, facilitating their natural
146 behavioral patterns and minimizing extraneous stress.

147 ***Animal model 2. Oral voluntary morphine consumption and spontaneous withdrawal syndrome***

148 ***precipitation by morphine deprivation:*** Just-weaned three-week-old female Wistar rats weighing 50-
149 65 g were single-housed under a constant temperature, with a 12-hour light/dark cycle, and
150 unrestricted access to standard rat chow and one bottle containing 0.15 mg/mL quinine hydrochloride
151 (Sigma-Aldrich) as the only water source to induce an adaptation to bitterness, facilitating the
152 subsequent oral voluntary morphine consumption as previously described (38).

153

154 All animal protocols were approved by the Committee for Experiments with Laboratory Animals at the
155 Universidad del Desarrollo (DCIM-2021/02). The brain microdialysis assays were approved by the
156 Animal Care Committee from the University of Valencia. The studies were conducted in accordance
157 with Spanish laws (RD 53/2013) and the European Directive (EC 2010/63).

158

159 Morphine administration and evaluation of withdrawal syndrome

160 ***Animal model 1: Subcutaneous morphine administration and withdrawal syndrome precipitation by***

161 ***naloxone.***

162 In this model, morphine dependence was developed in Wistar rats by implanting osmotic mini-pumps
163 subcutaneously (2ML2 ALZET®). These mini-pumps were loaded with morphine hydrochloride
164 (Sanderson Laboratory) to deliver a dose of 10 mg/kg/day, facilitating a steady and continuous release
165 of the drug (5 µl/hr) over seven days, following the manufacturer instructions. Controls animals were
166 implanted subcutaneously with osmotic mini-pumps releasing a saline solution. This model simulates

167 a state of morphine dependence in rats, mirroring the constant exposure to the drug in human
168 scenarios (39).

169 Three days post-pump implantation, the morphine-treated animals were randomly divided into two
170 groups. One group received simultaneously an intranasal (25 µg of protein) and intravenous (25 µg of
171 protein) dose of secretome derived from 1×10^6 preconditioned human MSCs (n=8), while the other
172 group received the vehicle (n=8). Both doses were administered in a volume of 160 µL. Animals of the
173 control group (saline-treated) received intranasal and intravenous administration of vehicle. The dual-
174 route administration aims to simultaneously targeted the central and peripheral alterations induced
175 by morphine dependence.

176 After seven days of continuous morphine exposure, rats were intraperitoneally injected with 5 mg/kg
177 of the μ -opioid receptor antagonist naloxone (Sigma-Aldrich) dissolved in 0.9% saline. Naloxone
178 administration triggered the sudden onset of a severe withdrawal syndrome (40), allowing for a
179 controlled observation and analysis of the somatic signs associated with opioid withdrawal.

180 Immediately after naloxone injection, each animal was placed in a glass beaker (300 mm height and
181 180 mm diameter) and monitored for a range of withdrawal symptoms for 30 minutes. These
182 symptoms included weight loss, percentage of the area covered by feces, and somatic signs including
183 jumps, ventral/dorsal flexes, stretching, chewing, burrowing, wet dog shakes, and forepaw tremors.
184 Additionally, vocalizations were recorded when the animal was held at the end of the induced
185 withdrawal syndrome. The behavioral monitoring was conducted by two investigators who were
186 blinded to the treatment conditions. The timeline of the experiments is depicted in **Supplementary**
187 **Figure 1A.**

188
189 ***Animal model 2: Oral voluntary morphine consumption and spontaneous withdrawal syndrome***
190 ***precipitation by morphine abstinence.***

191 In this model, Wistar rats voluntarily ingested morphine over a three-week period, leading to a state
192 of morphine dependence as previously described (38), resembling the clinical situation of chronic
193 morphine dependent patients . Briefly, following an initial seven-day exposure to 0.15 mg/mL quinine

hydrochloride as the only water source, the animals transitioned to a two-week regimen where they were given a choice between two bottles. One bottle contained quinine hydrochloride (0.15 mg/mL) and the other a solution of morphine sulfate (Oramorph, Molteni Farmaceutics; 0.15 mg/mL), both dissolved in tap water. After this period, the quinine bottle was removed, leaving the animals with a choice between tap water and morphine sulfate (0.15 mg/mL) for an additional week. A control group of animals had only access to water. To prevent side preference, the positions of the bottles were alternated daily. Morphine intake and preference, overall fluid consumption, and body weight were recorded daily. Unlike Model 1, where the withdrawal was precipitated by an μ -opioid receptor antagonist, in this model a withdrawal syndrome was induced by discontinuation of morphine access after three weeks of morphine exposure. On the same day, rats were administered simultaneously with intranasal and intravenous doses (same doses as Model 1) of either secretome or saline, depending upon the experimental group (**Supplementary Figure 1B**). This allowed us to evaluate the spontaneous manifestation of somatic signs of morphine withdrawal, which were recorded for 30 minutes 48 hours later. This approach simulates the natural course of withdrawal that would occur in an individual dependent on morphine who suddenly discontinues drug use, allowing us to test the effect of secretome administration during this stage.

In both animal models, a deprivation score was calculated based on the frequency of somatic withdrawal signs. This score was derived from a graduated scoring system for each parameter observed during a 30-minute evaluation period as previously reported (41, 42).

Isolation, expansion and characterization of human adipose tissue-derived MSCs.

Human mesenchymal stem cells (hMSCs) were obtained from freshly harvested subcutaneous adipose tissue, a rich source of hMSCs (36, 43). The tissue was collected from patients undergoing cosmetic liposuction at Clínica Alemana, Chile. Prior to the procedure, written informed consent was obtained. The entire process was conducted under protocols approved by the Faculty of Medicine Ethics Committee at Clínica Alemana-Universidad del Desarrollo, Santiago, Chile.

hMSCs were isolated from the adipose tissue as previously described (34, 43). After two subcultures, the cells were characterized based on the criteria proposed by the International Society for Cellular Therapy (44), evaluating their phenotypic profile by surface marker expression and adipogenic, osteogenic and chondrogenic differentiation capabilities, thereby confirming the cells' identity as hMSCs (34, 43).

226

hMSC preconditioning and secretome production.

hMSCs at the third passage and 75% confluency were subjected to a preconditioning process. This process was designed to increase the abundance of potent anti-inflammatory and antioxidant molecules released by the cells as previously described (34). Preconditioning was achieved by incubating the cells in minimum essential medium (α -MEM, Gibco) supplemented with 10% fetal bovine serum (FBS, HyClone) plus 10 ng/ml TNF- α (R&D System) and 15 ng/ml IFN- γ (R&D System) for 40 hours (36, 43). After preconditioning, the hMSCs were exhaustively washed to remove the pro-inflammatory cytokines and were cultured for an additional 48 hours in α -MEM, without FBS and phenol red. The culture medium (secretome) was then collected and centrifuged at 400g for 10 minutes to remove any detached cells. The supernatant was subjected to a second centrifugation at 5,000g for 10 minutes to eliminate any residual cell debris. The secretome was then filtered using 0.22 μ m filters and concentrated to fifty times its original volume using 3 kDa cutoff filters (Millipore). The protein concentration was determined using BCA protein assay kit (Thermo Scientific), and aliquots of the secretome were stored at -80°C pending further applications.

241

Non-invasive administration of secretome derived from preconditioned hMSCs.

Before secretome administration, rats were anesthetized by an intramuscular administration of ketamine (60 mg/kg) and acepromazine (4mg/kg) (45), and then positioned in supine position. A total of 160 μ l of the secretome, containing 25 μ g of proteins derived from 1×10^6 preconditioned hMSCs, was administered intranasally. This was done by 20 μ l droplets, alternately administered into each nostril with a pipette tip over a period of 40 minutes (four times into each nostril) (36). Control animals

received the same volume of saline solution. In addition to the intranasal route, the secretome was also administrated intravenously. This was performed immediately after intranasal delivery, with animals receiving a tail vein injection of 160 μ l of secretome containing 25 μ g of proteins derived from 1×10^6 preconditioned hMSCs. The control group received an equivalent volume of saline solution (23).

Assessment of morphine-induced neuroinflammation.

To evaluate neuroinflammation, we quantified the density of astrocytes and microglial cells in the hippocampus and the nucleus accumbens of rats, as previously described (34, 46). Rats were anesthetized by inhalation of 4% sevoflurane vapors (Baxter), and then intracardially perfused with 0.1 M PBS (pH 7.4) before euthanasia for brain sample collection.

Astrocyte and microglial density were evaluated by double-labeling immunofluorescence against the astrocyte marker glial fibrillary acidic protein (GFAP), and the microglial marker ionized-calcium-binding adaptor molecule 1 (Iba-1) in coronal 30 μ m thick cryo-sections of the hippocampus and nucleus accumbens, following previously reported methods (47). In brief, the coronal sections were washed with 0.1 M PBS, and blocked for 1 hour with a blocking solution (0.3% Triton X-100, 0.1% BSA, and 10% normal goat serum in PBS). The sections were then incubated overnight at 4 °C with a primary rabbit monoclonal anti-IBA-1 antibody (cat#019-19741, Wako) at a dilution of 1:500 and with a primary mouse monoclonal anti-GFAP antibody (cat#G3893, Sigma-Aldrich) at a dilution of 1:500 in the blocking solution. After this incubation, the sections were rinsed with PBS containing 0.3% Triton X-100, thereafter incubated for 2 hours at room temperature in the dark, with a goat anti-rabbit secondary antibody (Alexa Fluor 594, Thermo Fisher Scientific) at a dilution of 1:500 and a goat anti-mouse secondary antibody (Alexa Fluor 488, Thermo Fisher Scientific) at a dilution of 1:500 in the blocking solution and counterstained with 4,6 diamino-2-phenylindol (DAPI, Thermo Fisher Scientific, 0.02 M; 0.0125 mg/mL) for nuclear labeling. Microphotographs were taken from the *Stratum Radiatum* of the hippocampus and from the nucleus accumbens (NAc) using a confocal microscope (Olympus FV10i). The area analyzed for each stack measured 0.04 mm², with the thickness (Z axis) being recorded

274 for each case. The density of GFAP-positive astrocytes and Iba-1-positive microglial cells was
275 determined using the FIJI image analysis software as previously reported (46, 47).

276

277 **Assessment of morphine-induced oxidative stress.**

278 To evaluate oxidative stress in the hippocampus of rats subjected to morphine exposure, with or
279 without hMSC-derived secretome administrations and under control condition (no morphine), we
280 measured the ratio of oxidized glutathione normalized by total protein (GSSG/protein) and the level
281 of a lipid peroxidation marker (malondialdehyde, MDA, levels). Both measurements serve as indicators
282 of oxidative stress (23, 46, 48). To evaluate the GSSG/protein ratio, we homogenized the hippocampus
283 using a potassium buffer solution supplemented with 5 mM EDTA (pH 7.4). This homogenate was
284 centrifuged at 18,000g for 20 minutes, after which an aliquot was removed from the supernatant for
285 protein measurement, before adding trichloroacetic acid (Sigma-Aldrich, T0699) for protein
286 precipitation. Then, the homogenate underwent a second round of centrifugation at 18,000g for 15
287 minutes. 20 μ L of the resultant supernatant was treated with 0.5 μ L of 2-vinylpyridine (Sigma-Aldrich,
288 100-69-6) to chemically mask reduced glutathione (GSH) (49), preventing the initial binding of the thiol
289 group from GSH with sulfhydryl reagent DTNB (Sigma-Aldrich), as this union exhibits absorbance at
290 412nm. Any excess of 2-vinylpyridine was neutralized using triethanolamine. To evaluate the amount
291 of GSSG, we first incubated the sample with β -NADPH (Sigma-Aldrich, N1630) and 5,5'-dithiobis-(2-
292 nitrobenzoic acid) (DTNB). Then, GSSG in the sample was converted into GSH by the addition of
293 glutathione reductase (Sigma-Aldrich, G3664) and incubated at 37 °C in a microplate reader (Multiskan
294 Sky, Thermo). GSH levels were measured by absorbance at 412 nm and the GSSG concentration in the
295 sample was obtained using a calibration curve. Protein levels were measured by the BCA method
296 (Thermo).

297

298 Lipid peroxidation was evaluated by measuring the formation of MDA, a product of lipid peroxidation.

299 The MDA levels were assessed using the Lipid Peroxidation assay kit (Sigma-Aldrich), following a
300 previously reported method (23, 48) and expressed as nmol MDA/mg protein.

301

302 Surgical implantation of microdialysis probe.

303 Under isoflurane anesthesia, the rat was positioned in a stereotaxic apparatus (Stoelting). After
304 disinfecting the skin, a small incision was made over the skull, and the site was treated with 3%
305 lidocaine gel for analgesia. The Lambda and Bregma points on the skull were identified to determine
306 the placement of the microdialysis probes. Using these reference points, bilateral vertical concentric-
307 style microdialysis probes were implanted into the NAc (anteroposterior: +1.5 mm, mediolateral: ± 1.6
308 mm, dorsoventral: -8.0 mm from Bregma) (50). Holding screws were strategically inserted at two
309 random points near the NAc cranial placement for enhanced stability. The probes were secured with
310 dental cement, and the incision was sutured. After the surgery, the rats were allowed to recover in
311 their cages. A 2ML2 ALZET® systemic continuous infusion pump, loaded with morphine or vehicle as
312 described above for Animal Model 1, was also implanted during this procedure.

313

314 Assessment of glutamate levels by no-net-flux microdialysis.

315 To evaluate the effect of secretome administration on glutamate levels in the context of morphine
316 withdrawal syndrome, rats were divided into control, morphine-saline, and morphine-secretome. As
317 described above for Animal Model 1, the control group was implanted with a mini-pump filled with
318 saline, and the morphine-saline and morphine-secretome groups were implanted with mini-pumps
319 containing morphine to deliver a constant dose of morphine (10 mg/kg/day) over seven days. Three
320 days after pump implantation, the morphine-secretome group received intranasal and intravenous
321 doses of hMSCs-secretome, while the other two groups received saline. Seven days post-implantation,
322 the microdialysis process was started. Probes were perfused with artificial cerebrospinal fluid (aCSF)
323 at a rate of 2.5 $\mu\text{L}/\text{min}$. After an initial stabilization period of at least one hour, samples were collected
324 every 20 minutes to establish baseline glutamate levels. This was followed by the no-net-flux
325 technique, which was used to precisely determine the extracellular glutamate levels in the
326 implantation site by infusing increasing concentrations of glutamate (0.5 μM ; 5 μM ; 10 μM), with
327 samples collected for each concentration (following the protocol reported by Shen et al., 2014 (29)).
328 The placement of the cannula was confirmed post-operation using cresyl violet staining

329 **(Supplementary Figure 2).** Only animals with correct microdialysis cannula placements were included
330 in the data analyses.

331
332 To quantify glutamate concentration in the collected microdialysis samples, we employed a High-
333 Performance Liquid Chromatography (HPLC) system equipped with a fluorescence detector (Perkin-
334 Elmer series 200 HPLC System and Perkin Elmer Series 200 Fluorescence Detector for HPLC) for the
335 measurement of the fluorescent product of glutamate derivatization. The system was set up with an
336 excitation wavelength of 350 nm (51) and an emission wavelength of 452 nm, reported as optimal
337 parameters for glutamate determination by Perucho et al., 2015 (52). The mobile phase A was
338 composed of 0.1 M sodium acetate adjusted to pH 6.9 with acetic acid, 2.5% methanol, and 2.5%
339 tetrahydrofuran. The mobile phase B was 100% methanol. Mobile phase 1 was isocratically eluted at
340 1.5 mL/min, thereafter the mobile phase A was connected and allowed to equilibrate for 1 hour before
341 the start of the runs. For derivatization, a cocktail was prepared containing 27 mg of 2-ophtalaldehyde
342 (OPA) (Sigma-Aldrich), dissolved in 500 μ L of absolute ethanol, 4.5 mL of borate buffer (pH 9.3), and
343 20 μ L of β -mercaptoethanol (Sigma-Aldrich) (53). The samples were mixed 1:1 with the derivatization
344 cocktail and a 30 μ L aliquot of the derivatized sample was injected into the HPLC system. The
345 glutamate-derivative was retained using a reverse-phase HPLC column C18, 5 μ m; 50 $\times$ 4.6 mm
346 (Ascentis® Express). Three minutes post-injection, a gradient was manually initiated by connecting a
347 bottle with 65% mobile phase A and 35% mobile phase B. After 1 minute, the mobile phase was
348 switched to 10% A and 90% B, and after another minute, it was changed to 100% A. The run was
349 completed after 15 minutes, allowing the pressure system to return to the baseline level. The
350 glutamate concentration in each sample was determined by comparing the peak heights with an
351 external standard curve. This method allowed for precise quantification of glutamate levels in the NAc,
352 providing insights into the effects of morphine withdrawal and hMSCs-derived secretome treatment
353 on glutamatergic balance in this key brain region (52).

354
355 **Determination of xCT and GLT-1 glutamate transporter levels in nucleus accumbens.**

356 Finally, we measured the protein levels of glutamate transporters GLT-1 and xCT in the NAc using
357 Western blot. For this, proteins from NAc were extracted using T-PER lysis buffer (Thermo-Fisher)
358 supplemented with a protease inhibitor. 25 µg of protein were loaded to each lane. The GLT-1 protein
359 was identified using a guinea pig anti-GLT-1 primary antibody (Cat AB1783, Millipore, 1:500 dilution)
360 and an IRDye 800CW donkey anti-guinea pig secondary antibody (Cat 925–32411, LI-COR, 1:10,000
361 dilution). For xCT detection, a rabbit anti-xCT primary antibody (Cat AB175186, Abcam, 1:500 dilution)
362 was used in conjunction with an IRDye 800CW donkey anti-rabbit secondary antibody (Cat 926–32213,
363 LI-COR, 1:10,000 dilution). β-actin, detected using a mouse anti-β-actin primary antibody (Cat sc-47778
364 Santa Cruz Biotechnology, 1:200 dilution) and an IRDye 800CW goat anti-mouse secondary antibody
365 (Cat 926–32210, LI-COR), served as the loading control. The reactive bands were captured with the
366 Odyssey Imaging System (LI-COR), and the intensities were quantified using Image Studio Lite 5.2
367 software.

368

369 **Statistical Analysis.**

370 All statistical evaluations were conducted using GraphPad Prism software (9.2.0 version). Data are
371 presented as means ± SEM. The Shapiro-Wilk test was used to verify the normality of the distribution
372 for all experimental data. Bartlett's test was employed to confirm the homogeneity of variances
373 between groups, with a p-value > 0.05 indicating equal variances. Upon satisfying these assumptions
374 of normality and homogeneity, either one-way or two-way analysis of variance (ANOVA) was utilized,
375 depending on the experimental conditions. Following the ANOVA, a Tukey post-hoc test was
376 performed to examine the differences between experimental groups. A p-value < 0.05 was considered
377 to indicate statistical significance. To facilitate understanding and interpretation, the complete
378 statistical analyses are provided in the figure legends.

379

380

381

382

383 RESULTS

384

385 Effects of secretome administration on somatic signs of morphine dependence and withdrawal score 386 in the naloxone precipitated withdrawal model.

387 Previous work demonstrated that the intravenous and intranasal administration of secretome derived
388 from preconditioned hMSCs significantly reduced oral morphine consumption in an animal model of
389 voluntary morphine intake (23). Building on this observation, we sought to determine whether the
390 hMSC-derived secretome could also alleviate the withdrawal stage of opioid abuse. To this end, we
391 comprehensively evaluated the somatic signs of withdrawal syndrome in morphine-treated Wistar
392 rats. Towards this aim, animals were implanted with subcutaneous osmotic mini-pumps that delivered
393 morphine (10 mg/kg/day) for seven days and treated both systemically and intranasally with MSC-
394 derived secretome or vehicle three days after pump implantation. On day seven, withdrawal syndrome
395 was induced by the intraperitoneal administration of the μ -opioid antagonist naloxone. The
396 quantification of somatic withdrawal signs showed significant differences between the morphine-
397 vehicle and morphine-secretome treated groups compared to the no-morphine control group (**Figure**
398 **1**). These signs include weight difference before and after the induction of the withdrawal syndrome
399 ($p < 0.0001$, One-way ANOVA followed by Tukey's post-hoc test) (**Figure 1A**), the area covered by feces
400 ($p < 0.0001$, One-way ANOVA followed by Tukey's post-hoc test) (**Figure 1B**), and jump events ($p < 0.05$,
401 One-way ANOVA followed by Tukey's post-hoc test) (**Figure 1C**). When comparing both groups
402 administered with morphine, the morphine-secretome group displayed a significant reduction in two
403 somatic signs: stretching events ($p < 0.05$, One-way ANOVA followed by Tukey's post-hoc test) (**Figure**
404 **1E**) and chewing events ($p < 0.05$, One-way ANOVA followed by Tukey's post-hoc test) (**Figure 1F**)
405 compared with the morphine-vehicle group.

406

407 We also adapted a somatic withdrawal score based on the frequency of withdrawal symptoms
408 (**Supplementary Table 1**), as it has been previously reported that a withdrawal score provides a robust
409 evaluation for the severity of withdrawal signs (41, 42). With this analytical methodology, we observed

that rats exposed to morphine and treated with the vehicle displayed a significantly elevated somatic withdrawal score compared to the control rats ($p < 0.01$, One-way ANOVA followed by Tukey's post-hoc test) (**Figure 2**). However, this trend was notably altered following the administration of the hMSC-derived secretome. Rats that were administered with secretome simultaneously intranasally and intravenously, exhibited a significant reduction in naloxone-triggered withdrawal score compared to the group treated with the vehicle ($p < 0.05$, One-way ANOVA followed by Tukey's post-hoc test) (**Figure 2**).

Impact of secretome administration on astrocyte and microglial density in the hippocampus.

Considering the pivotal role of the hippocampus in associative memory networks, the encoding and consolidation of novel environmental information, and its documented contribution to the development of drug-seeking memory behavior (54, 55), we evaluated neuroinflammation in this brain region in the morphine antagonist-triggered withdrawal model prior to naloxone administration. This evaluation holds particular relevance in light of evidence indicating the occurrence of hippocampal neuroinflammation caused by naloxone administration in a morphine dependence rat model (56). As expected, rats that received morphine exhibited a significant increase in astrocyte density compared to control rats ($p < 0.01$, One-way ANOVA followed by Tukey's post-hoc test) (**Figure 3A and 3C**). A similar trend was observed for microglial density. However, this increase did not reach statistical significance ($p = 0.0828$, One-way ANOVA followed by Tukey's post-hoc test) (**Figure 3B and 3D**). By contrast, rats that received morphine but were treated with secretome showed a significant reduction in the increased astrocyte density in the hippocampus ($p < 0.05$, One-way ANOVA followed by Tukey's post-hoc test) (**Figure 3A and 3C**), while microglia density tended to decrease (**Figure 3B and 3D**).

Effects of secretome administration on hippocampal oxidative stress markers.

Given the substantial evidence of the presence of oxidative stress during chronic morphine consumption (23, 57), and the withdrawal stage (19, 58), we evaluated the presence of hippocampal oxidative stress in the morphine antagonist-triggered withdrawal model prior to naloxone

administration. However, the evaluation of oxidative stress markers, including GSSG levels and MDA levels, did not reveal significant changes during the seven-day morphine exposure in the morphine-vehicle group compared to the control group (**Figure 4A and 4B**). Similarly, secretome administration only trended to reduce hippocampal MDA levels ($p=0.0867$, One-way ANOVA followed by Tukey's post-hoc test) compared to animals treated with vehicle (**Figure 4B**), suggesting that seven days of morphine exposure is not enough to induce a significant increase in hippocampal oxidative stress.

Impact of secretome administration on astrocyte and microglial density in the nucleus accumbens.

We subsequently centered our focus on the nucleus accumbens inflammatory state. In this brain area, animals that received morphine showed a significant increase in astrocyte (**Figure 5A and 5C**) and microglial density (**Figure 5B and 5D**) compared to that in control rats ($p<0.01$, One-way ANOVA followed by Tukey's post-hoc test). Importantly, rats that received morphine and were treated with secretome showed a significant reduction in the increased astrocyte (**Figure 5A and 5C**) and microglial density (**Figure 5B and 5D**) ($p<0.05$, One-way ANOVA followed by Tukey's post-hoc test).

Effects of secretome administration on the modulation of nucleus accumbens glutamate levels and the expression of glutamate transporters GLT-1 and xCT.

Following the observation that secretome treatment promoted a reduction in NAc neuroinflammatory markers, we examined the effects of secretome administration on extracellular glutamate levels in this brain region. A one-way ANOVA revealed a significant reduction versus no-morphine controls in both the basal glutamate levels (**Figure 6A**) and in the no-net-flux glutamate levels (**Figure 6B**) in the NAc for both the morphine-vehicle and morphine-secretome treated animals ($p<0.0001$, One-way ANOVA followed by Tukey's post-hoc test) compared to the control group, suggesting a reduced spillover of extracellular glutamate. No significant difference was observed among glutamate levels measured in morphine exposed groups treated with secretome or vehicle ($p<0.05$, One-way ANOVA followed by Tukey's post-hoc test) (**Figure 6A and 6B**). However, a significant increase in the slope of the no-net-

463 flux curve was observed in the morphine-secretome group compared to the control groups (**Figure**
464 **6C**), which could indicate a modified glutamate transport dynamic.

465 The role of glial glutamate transporters in controlling glutamate uptake has been previously associated
466 with the emergence of morphine dependence (28, 59, 60). However, we did not observe any significant
467 changes either in the levels of the glutamate transporters GLT-1 (**Figure 7A and 7B**) or the xCT system
468 (**Figure 7C and 7D**) in NAc homogenates among all experimental groups.

469
470 **Effects of secretome administration on somatic signs of morphine dependence and withdrawal score**
471 **following deprivation after chronic oral voluntary morphine consumption.**

472 Finally, we examined the effects of the intravenous and intranasal administration of secretome on the
473 somatic signs of spontaneous withdrawal syndrome in an animal model of chronic voluntary oral
474 morphine consumption, 48 hours after morphine deprivation. This time point was selected based on
475 clinical reports suggesting that the peak of withdrawal symptoms typically occurs between 36 to 72
476 hours following the last opioid administration (31). Furthermore, another study in rats detected
477 morphine levels below the quantitative limits but above background noise 48 hours after
478 subcutaneous morphine administration (61). At this time point, it is expected that the concentration
479 of the opioid has diminished to less than 6.25% of the initial dose (62). For this, we used a recently
480 reported animal model of opioid dependence in which young Wistar rats were initially acclimated to a
481 bitter taste by exposing them to a bitter quinine solution as their only fluid source for seven days (38).
482 After this period, rats were given a free choice between quinine (15 mg/ml) or morphine (15 mg/ml)
483 solutions (two-bottle choice) for two weeks. During this time, rats exhibited a high morphine
484 consumption rate of 20.2 ± 2.1 mg/kg/day. Finally, rats were offered a choice between a morphine
485 solution (15 mg/ml) and water for one additional week. This led to a voluntary morphine intake of 16.8
486 ± 2.2 mg/kg/day (**Supplementary Figure 3A**), with a preference for the morphine solution over water
487 exceeding 90% (**Supplementary Figure 3B**). After three weeks of voluntary morphine intake, animals
488 were both deprived of morphine and treated with an intravenous and intranasal administration of
489 secretome or vehicle. Forty-eight hours later, the withdrawal signs were evaluated for 30 minutes. We

490 observed that upon withdrawal, morphine-exposed rats treated with the vehicle showed a significant
491 increase in different somatic signs compared to only water exposed animals, including weight
492 difference ($p < 0.05$, One-way ANOVA followed by Tukey's post-hoc test) (**Figure 8A**), the area covered
493 by feces ($p < 0.001$, One-way ANOVA followed by Tukey's post-hoc test) (**Figure 8B**), the number of
494 ventral/dorsal flexions ($p < 0.05$, One-way ANOVA followed by Tukey's post-hoc test) (**Figure 8D**), the
495 number of wet dog shakes ($p < 0.001$, One-way ANOVA followed by Tukey's post-hoc test) (**Figure 8G**),
496 and the number of forepaw tremors ($p < 0.05$, One-way ANOVA followed by Tukey's post-hoc test)
497 (**Figure 8H**). Contrasting the above, secretome-treated animals showed a significant reduction in many
498 of the morphine-induced withdrawal signs, including weight difference ($p < 0.05$, One-way ANOVA
499 followed by Tukey's post-hoc test) (**Figure 8A**), the area covered by feces ($p < 0.001$, One-way ANOVA
500 followed by Tukey's post-hoc test) (**Figure 8B**) and the number of wet dog shakes ($p < 0.05$, One-way
501 ANOVA followed by Tukey's post-hoc test) (**Figure 8G**) compared to vehicle treated animals.
502 Importantly, the combined withdrawal score of morphine-secretome treated animals was markedly
503 lower than that for morphine-vehicle treated animals ($p < 0.05$, One-way ANOVA followed by Tukey's
504 post-hoc test) (**Figure 9**).
505 The combined above findings underscore the potential therapeutic role of the secretome in reducing
506 symptoms of morphine withdrawal.

DISCUSSION

508

509 The present study examines the effects of simultaneous systemic and intranasal administration of
510 secretome derived from preconditioned human mesenchymal stem cells on morphine-induced
511 withdrawal syndrome in rats. The findings encompass behavioral, cellular, and molecular aspects that
512 have been previously described to be altered by chronic morphine administration (38). In both animal
513 models tested, we observed that somatic withdrawal signs were heterogeneously distributed,
514 indicating that some animals exhibit a significant increase in some of the analyzed parameters, while
515 others show fewer alterations on these parameters. This pattern was noted across all three studied
516 groups: control, morphine-vehicle, and morphine-secretome. This variability in behavior has been
517 previously reported, and is a primary reason for the literature to present a wide range of withdrawal
518 parameters to be scored (7, 63). Consequently, we consolidated these behavioral alterations into a
519 somatic withdrawal score, which was validated based on previous studies (41, 42) and adapted
520 considering the intensity and frequency of each parameter observed in our study. In both animal
521 models, secretome administration markedly reduced the withdrawal score.

522

523 Previous reports have established that the administration of secretome derived from hMSCs can
524 reduce chronic consumption and relapse of substances of abuse, including alcohol (34, 36), nicotine
525 (36), and morphine (23). Our study pioneers in demonstrating that this biodrug can also alleviate the
526 morphine withdrawal syndrome in two animal models of morphine dependence. This is a major
527 finding, considering that the withdrawal syndrome often triggers the relapse into opioid consumption,
528 as needed to avoid the somatic signs induced by opioid deprivation (31, 63, 64), thus impeding
529 morphine discontinuation (65-67).

530

531 Noteworthy, we observed this positive outcome following the simultaneous intravenous and
532 intranasal administration of the hMSC-derived secretome. In our previous studies on alcohol and
533 nicotine addiction, we found that the intranasal administration of the hMSC-derived secretome
534 effectively reduced substance abuse (36). However, when evaluating the therapeutic effect of

secretome on opioid dependence, we incorporated an additional administration route, following findings from other studies (23, 68). One of these studies showed that the intravenous administration of MSCs significantly reduced morphine-induced inflammation in the spinal cord, a peripheral alteration associated with opioid tolerance (68). Moreover, a preclinical rat model of morphine exposure during six days showed an increase in plasma IL-1 β and IL-6 levels, but intriguingly, the same cytokines did not increase at brain level (69). There are studies that have highlighted persistent systemic inflammation in drug users even after complete abstinence (18). This inflammation has been characterized by strong inflammatory responses during the early stage of abstinence, which then gradually alleviate along with the withdrawal time. However, even after 12 months of abstinence, immune dysfunction still exists and may persist for longer times in heroin and methamphetamine users (18). Therefore, in this report, we opted for a simultaneous intranasal and intravenous administration of the hMSC-derived secretome. The strategy with a dual-route administration aims to comprehensively address these complex central and peripheral changes induced by morphine dependence, providing a more effective treatment strategy. However, it's important to note that while our dual-route administration strategy is based on previous research, direct evidence demonstrating its superior efficacy compared to a single-route administration is still needed.

In the animal model of continuous morphine administration, secretome administration effectively mitigated the neuroinflammation that developed before the naloxone-triggered withdrawal stage. This was evidenced by the full reversal of the morphine-induced increase in astrocyte density within the hippocampus and astrocyte and microglial density in nucleus accumbens, regions crucially associated with memory consolidation (70) and brain reward circuitry (71). The observed impact of secretome administration on astrocyte and microglial density in morphine-dependent rats underscores a potential mechanism through which the secretome may exerts its therapeutic effects. The increase in astrocyte and microglial density following morphine administration aligns with findings indicating that opioids can activate different pro-inflammatory pathways (22, 69, 72). This activation could potentially occur via direct downstream signaling of the μ -opioid receptor (73), as well as via the

562 activation of the TLR-4 signaling pathway, binding to the adaptor molecule MD-2, within the central
563 nervous system (22, 74). Recent studies have described the role of morphine to induce peripheral
564 inflammation, specifically by inducing dysbiosis and amplifying bacterial translocation from the
565 intestine to the liver and mesenteric lymph nodes (75-77). This cascade of events potentially triggers
566 a systemic pro-inflammatory state (78, 79), thereby underscoring a new mechanism of action in opioid
567 dependence that merits further exploration. These findings highlight the complex interplay among
568 opioid addiction, secretome treatment and neuroinflammation, providing information for future
569 studies to evaluate these mechanisms.

570
571 While there is compelling evidence suggesting that like many other drugs of abuse (24), morphine can
572 induce an increase in oxidative stress (57) in cultures of hippocampal neurons (80) and in hippocampal
573 tissue from rats exposed to morphine (81), we did not observe a corresponding increase in oxidative
574 stress biomarkers in the hippocampal tissue, evaluated both by determination of GSSG and MDA levels
575 after the seven-day morphine exposure in animal model 1. The reasons for these differences are not
576 clear but may be associated with several factors. Recent evidence suggests that the effects of
577 morphine on oxidative stress may be dose- and time-dependent (82-84). This is aligned with previous
578 findings in an animal model of voluntary morphine intake, in which four weeks of morphine
579 consumption induced a three-fold increase in GSSG/GSH ratio and MDA levels in the hippocampus
580 compared with rats drinking only water (23), increases that were fully normalized by hMSC-derived
581 secretome administration (23). Another possible explanation for the absence of increased oxidative
582 stress in our animal model could be attributed to sex differences observed in response to opioid
583 exposure (85-87). A significant proportion of the reports showing an increase in brain oxidative stress
584 are based on studies conducted in male rats (58, 88-92), unlike the present studies in female rats,
585 suggesting that sex of the animal might also be an important variable (93).

586
587 Neuroinflammation and oxidative stress self-potentiate each other (94), and both processes have been
588 associated with a reduction of the levels and activity of the key glutamate transporters GLT-1 and

589 system X_c^- (24-27), resulting in the imbalance of glutamatergic neurotransmission and to the
590 potentiation of withdrawal symptoms and drug relapse (29). Intriguingly, while the continuous
591 morphine administration reduced the levels of extracellular glutamate in the NAc (glutamate spillover),
592 the administration of secretome did not change the effect of continuous morphine treatment on
593 glutamate extracellular level in this brain region. We did observe a significant increase in the slope of
594 the no-net-flux glutamate determination induced by the secretome treatment, suggesting a potential
595 effect on glutamate elimination dynamics, however, it did not significantly affect the expression of the
596 glutamate transporters GLT-1 or system X_c^- . A lack of changes in glutamatergic transporters was not
597 expected, but can be explained as reports show that the alteration of the transporters levels depends
598 on the specific opioid treatment scheme. For example, heroin self-administration and withdrawal
599 reduces the levels and activity of GLT-1 and system X_c^- and promotes an increase of glutamate spillover
600 to the extracellular space in the NAc, opposite to the reduced spillover observed in the present study,
601 an effect that also contributed to opioid dependence as the normalization of glutamate transporters
602 levels reduced heroin reinstatement (29). On the other hand, GLT-1 mRNA levels were reduced in the
603 striatum of rats after 5 days of continuous morphine exposure via a subcutaneous pellet compared to
604 placebo controls but were increased 2 hours after naloxone-induced withdrawal (95). Another study
605 showed that hippocampal glutamate uptake was increased after 10 days of bidaily 10 mg/kg morphine
606 administration, then initially reduced after 2 hours of naloxone-induced withdrawal, increased again
607 after 12 hours, and finally returned to baseline levels after 48 hours (59). These results show that the
608 requirement of the normalization of glutamatergic neurotransmission to reduce the withdrawal
609 syndrome intensity is not completely understood. Likewise, in our previous report of the treatment
610 with intranasal and intravenous hMSC secretome that significantly reduced morphine oral self-
611 administration, it did not alter GLT-1 or xCT mRNA levels in the prefrontal cortex or nucleus accumbens
612 compared to vehicle treatment, despite significant normalization of neuroinflammation and brain
613 oxidative stress levels (23). Similarly, the lack of normalization of extracellular glutamate levels in the

614 present model suggests that the hMSC secretome treatment reduces the opioid withdrawal syndrome
615 through other mechanisms.

616
617 Finally, the observation that secretome administration also alleviates spontaneous withdrawal
618 symptoms and reduces withdrawal scores following chronic voluntary oral morphine intake further
619 strengthens the potential of the secretome as a therapeutic strategy for managing opioid withdrawal
620 symptoms. The implications of this finding for the role of hMSC-derived secretome in the treatment of
621 morphine dependence and withdrawal warrant further exploration.

622

623 **CONCLUSION**

624 Overall, findings indicate the generation of therapeutic benefit by the simultaneous intravenous and
625 intranasal administration of secretome derived from preconditioned hMSC for mitigating morphine-
626 induced withdrawal syndrome in two relevant animal models of morphine dependence. These effects
627 were associated with a reduction of morphine-induced neuroinflammation in the hippocampus and
628 nucleus accumbens. While our study provides significant insights, further investigation is necessary to
629 fully elucidate the mechanisms underlying the observed effects and to evaluate the therapeutic
630 feasibility of secretome administration in other models of opiate abuse disorders.

631

632 **ACKNOWLEDGEMENTS**

633 This work was supported by FONDECYT 1200287 grant to Fernando Ezquer, ACT210012 grant to
634 Fernando Ezquer and Paola Morales, the Spanish Ministerio de Ciencias e Innovación PID2019-
635 109823RB-100/MICINN/AEI/10.13039/501100011033 to Lucia Hipólito and 21212198 ANID Doctorate
636 Grant to Mauricio Quezada. The technical assistance of Ms. Catalina Vallejos and Mr. Jorge Ruiz is
637 greatly appreciated.

638

639 **CONFLICT OF INTEREST**

640 The authors declare no conflict of interest.

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642

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899 precipitated withdrawal. *Brain Res*. 2001 Jun 29;905(1-2):254-8. PubMed PMID: 11423104.
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FIGURES AND FIGURE LEGENDS

FIGURE 1

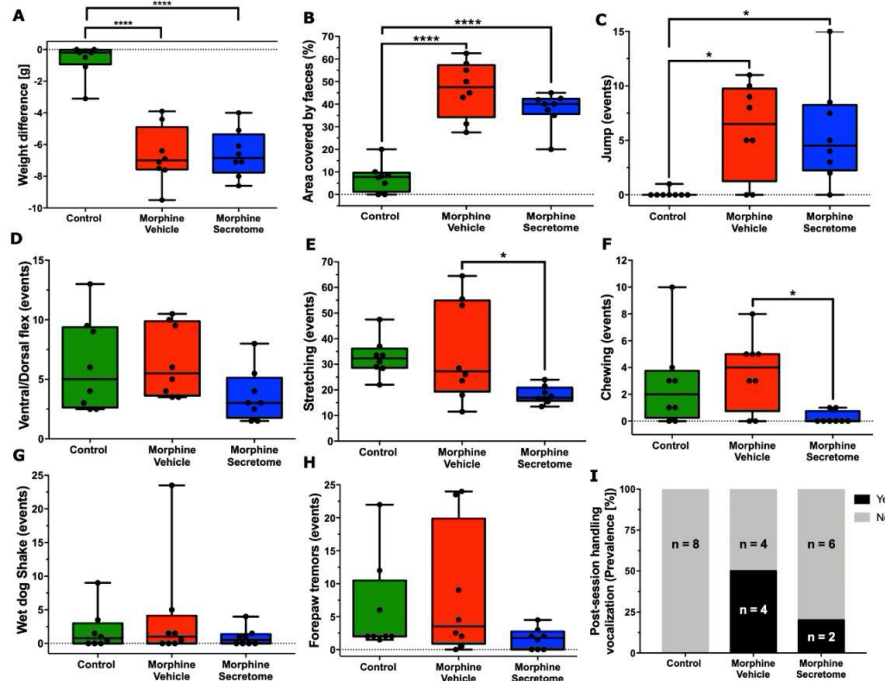


Figure 1: Effects of secretome administration on somatic signs of morphine dependence and

withdrawal score in the naloxone-precipitated withdrawal model. Eight-week-old female Wistar rats

were implanted subcutaneously with osmotic pumps delivering morphine at a concentration of 10

mg/kg/day for seven days. On day three, animals received an intranasal and intravenous

administration of MSC-derived secretome (25 μ g proteins derived from 1×10^6 preconditioned hMSCs)

or saline. Withdrawal syndrome was induced on day seven by the intraperitoneal administration of

naloxone (5 mg/kg), after which behavior was recorded for 30 minutes to gauge the severity of signs.

The following signs were evaluated: (A) weight differences, (B) area covered by feces, (C) jumps, (D)

ventral/dorsal flexes, (E) stretching, (F) chewing, (G) wet dog shakes, (H) forepaw tremors events, and

(I) post-session handling vocalization prevalence. Control animals received saline solution from a

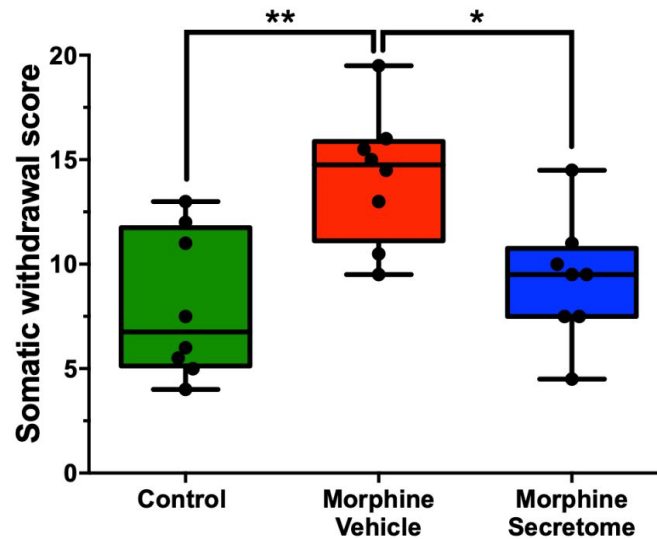
continuous infusion pump; n=8 per experimental condition. Data are presented as Min to Max.

*p<0.05; ****p<0.0001; Tukey's multiple comparison test for normally distributed data and Kruskal-

Wallis test for non-parametric data.

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FIGURE 2



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919 **Figure 2: Effects of secretome administration on the combined withdrawal score in the naloxone**
920 **precipitated withdrawal model.** The somatic withdrawal is depicted using a scoring system based on
921 previous studies (41, 42). Secretome treated rats showed a significant reduction in the somatic
922 withdrawal score compared to morphine-vehicle treated animals. Animals not exposed to morphine
923 were considered controls. Data are presented as Min to Max; n = 8 for each experimental condition.

924 *p<0.05; **p<0.01; Tukey's multiple comparison tests for normal distribution.

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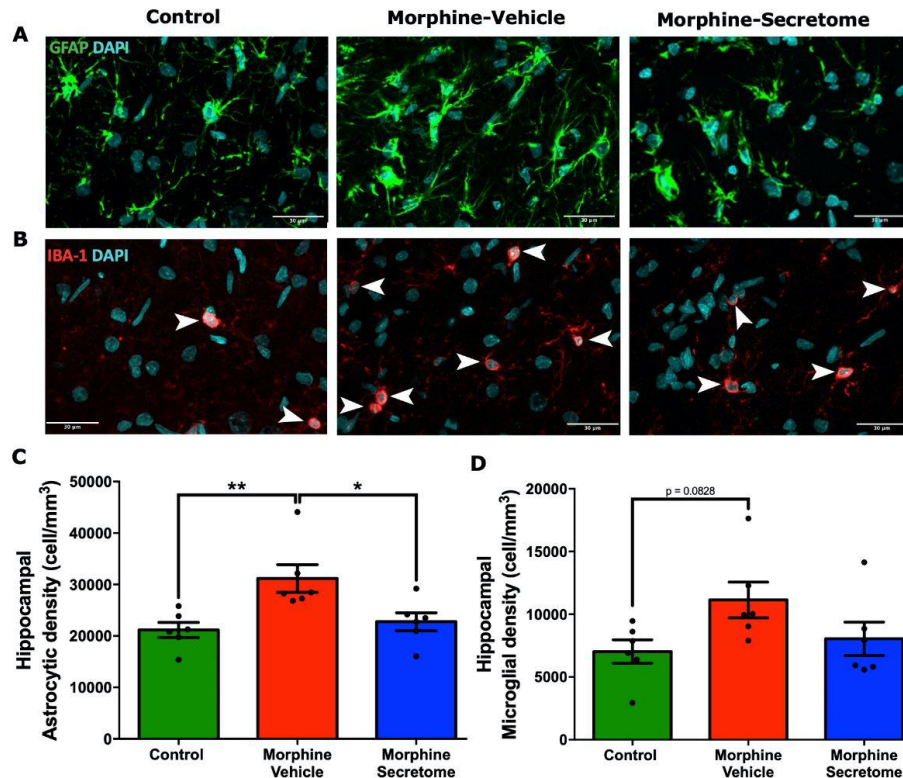
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FIGURE 3



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937 **Figure 3: Effect of secretome administration on hippocampal astrocyte and microglial density. (A and**938 **B) Representative confocal microphotographs of GFAP immunoreactivity (depicted in green, top panel)**939 **of hippocampal astrocytes and Iba-1 immunoreactivity (shown in red, indicated by white arrows,**940 **bottom panel) of hippocampal microglial cells. The nuclei were counterstained with DAPI (blue, nuclear**941 **marker); scale bar: 30 μ m. (C) Quantification of astrocyte density. (D) Quantification of microglial**942 **density. Rats implanted with a morphine pump and treated with vehicle exhibited a significant increase**943 **in astrocyte density, but not in microglial density, compared to control rats. A single, simultaneous**944 **intranasal and intravenous administration of secretome three days after morphine-pump implantation**945 **(morphine-secretome) significantly reduced astrocyte density compared to that in morphine-vehicle**946 **treated rats. Data are presented as mean $\pm$ SEM. n = 6 for each experimental condition. *p < 0.05,**947 ****p < 0.01 One-way ANOVA followed by Tukey's post-hoc test.**

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FIGURE 4

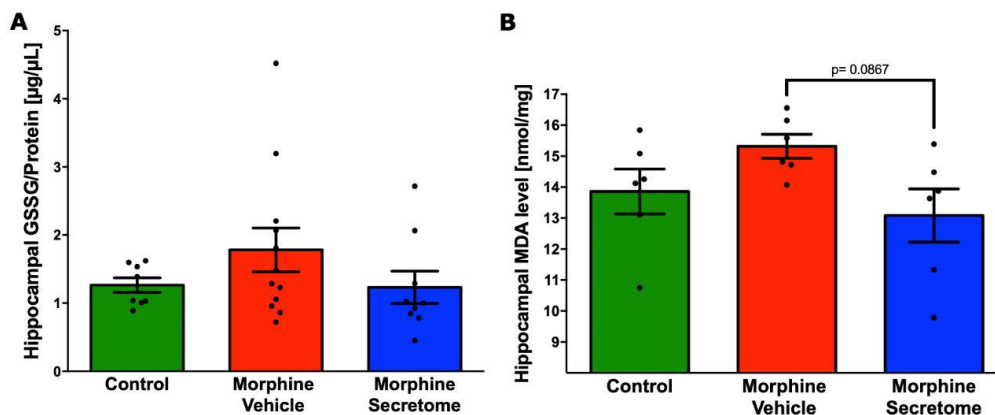
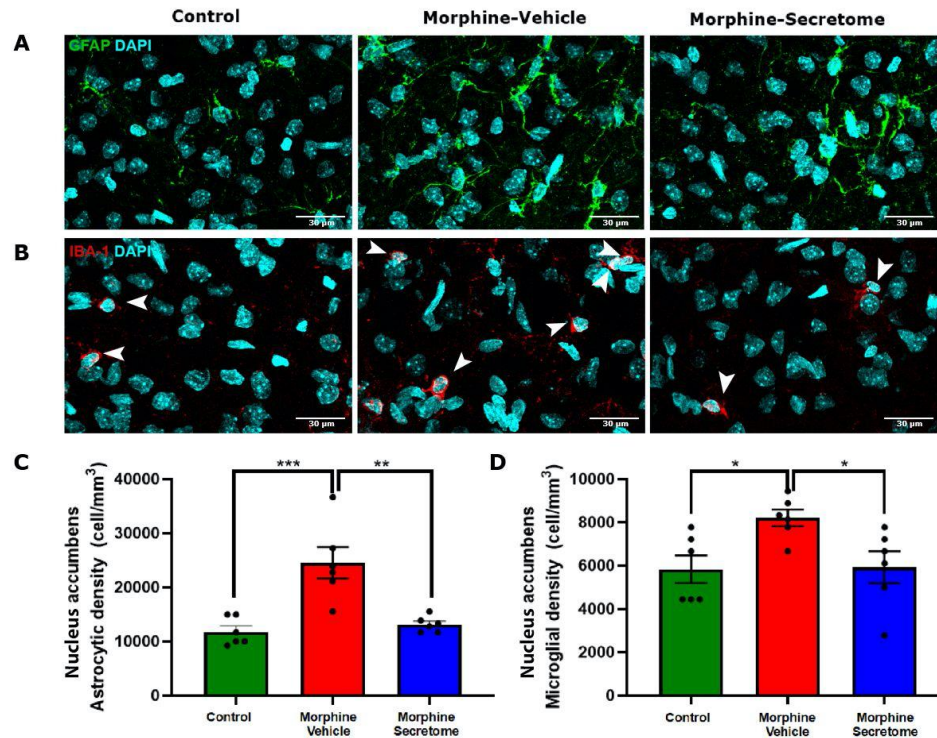


Figure 4: Evaluation of morphine administration and secretome administration on oxidative stress markers in the hippocampus. (A) GSSG/protein ratio in the hippocampus. (B) Malondialdehyde (MDA) levels in the hippocampus. Rats infused with morphine for seven days and treated with vehicle did not show a significant increase in GSSG/protein ratio or MDA levels compared to no-morphine control rats. A single, simultaneous intranasal and intravenous administration of secretome did not alter GSSG/protein ratio or MDA levels. Data are presented as mean $\pm$ SEM; n=6 to 8 for each experimental condition. One-way ANOVA followed by Tukey's post-hoc test.

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FIGURE 5

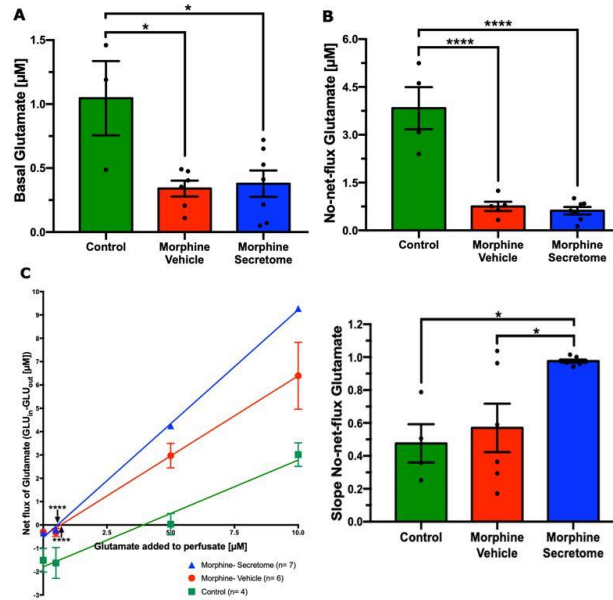


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971 **Figure 5: Effect of secretome administration on the morphine-induced increases in astrocyte and**
 972 **microglial density in nucleus accumbens. (A and B)** Representative confocal microphotographs of
 973 GFAP immunoreactivity (depicted in green, top panel) of hippocampal astrocytes and Iba-1
 974 immunoreactivity (shown in red, indicated by white arrows, bottom panel) of hippocampal microglial
 975 cells. The nuclei were counterstained with DAPI (blue, nuclear marker); scale bar: 30 μ m. **(C)**
 976 Quantification of astrocyte density. **(D)** Quantification of microglial density. Rats implanted with a
 977 morphine pump and treated with a vehicle exhibited a significant increase in astrocyte density and in
 978 microglial density, compared to control rats. A single, simultaneous intranasal and intravenous
 979 administration of secretome three days after morphine-pump implantation significantly reduced
 980 astrocyte and microglial density compared to vehicle treated rats. Data are presented as mean $\pm$ SEM.
 981 n = 6 for each experimental condition. *p<0.05, **p<0.01 One-way ANOVA followed by Tukey's post-
 982 hoc test.

983

FIGURE 6



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985 **Figure 6: Evaluation of secretome administration effects on glutamate levels in nucleus accumbens.**

986 Eight-week-old female Wistar rats were implanted subcutaneously with osmotic pumps delivering
 987 morphine at a concentration 10 mg/kg/day. In the same procedure rats underwent microdialysis probe
 988 implantation in the NAc. Control animals received saline-loaded pumps (n=4), while morphine-saline
 989 (n=6) and morphine-secretome (n=7) groups received morphine-loaded pumps (10 mg/kg/day). Three
 990 days post pump implantation, secretome or vehicle was administrated and seven days post pump
 991 implantation, no-net-flux microdialysis was used to determine NAc glutamate levels. Following a 1-
 992 hour stabilization period, samples were collected every 20 minutes for basal glutamate levels. Then,
 993 increasing concentrations of glutamate (0.5 μM , 5 μM , 10 μM) were perfused. **(A)** Quantification of
 994 basal glutamate concentrations average. **(B)** No-net-flux glutamate concentration. **(C)** No-net-flux
 995 plots (glutamate concentration set to enter the probe (GLU_{in}) – glutamate concentration output
 996 (GLU_{out}) v/s GLU_{in}) and quantification of the slopes obtained from no-net-flow curves. Both
 997 experimental groups exposed to morphine significantly reduced the extracellular glutamate
 998 concentration, compared to the control group. Data are presented as mean $\pm$ SEM. * $p < 0.05$,
 999 **** $p < 0.0001$, One-way ANOVA followed by Tukey's post-hoc test.

FIGURE 7

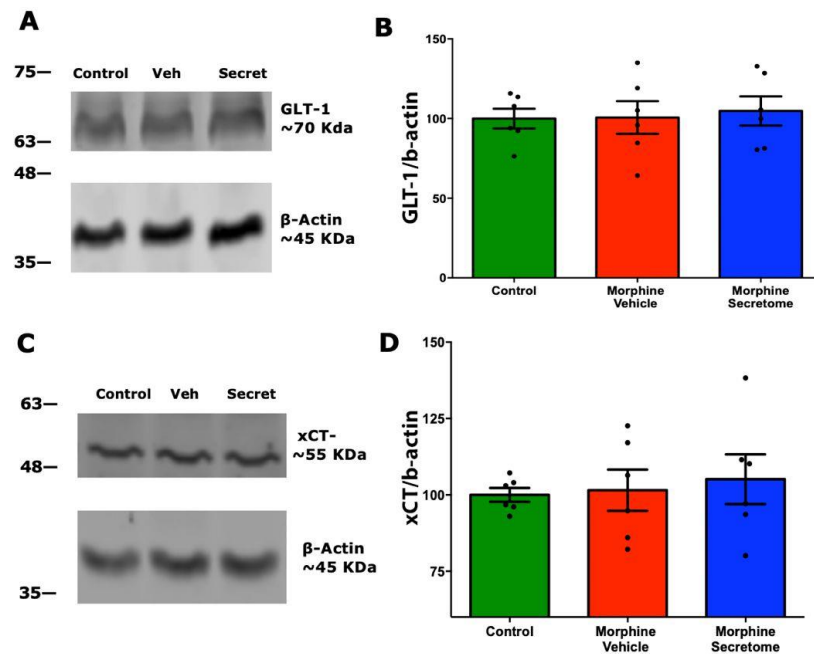
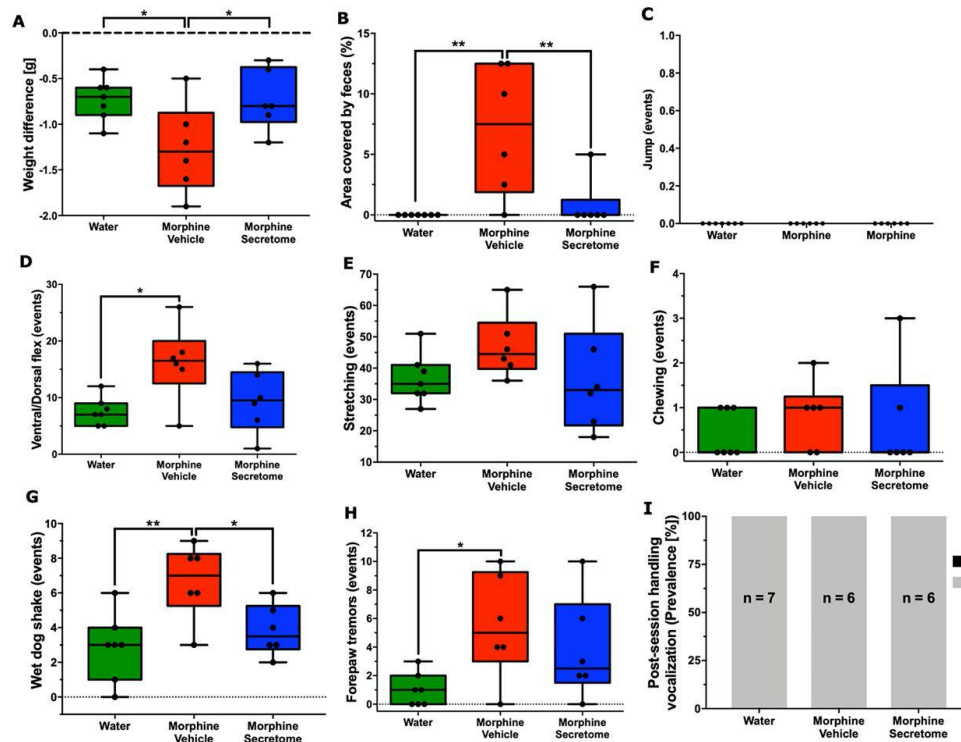


Figure 7: Evaluation of secretome effects on GLT-1 and xCT glutamate transporter levels in nucleus accumbens. (A) Representative images of Western Blot for the glutamate transporter GLT-1 in NAc. (B) Quantification of GLT-1 to β -actin levels in NAc. (C) Representative images of Western Blot for the glutamate transporter xCT in NAc. (D) Quantification of xCT to β -actin levels in NAc. No significant differences were observed in the expression of GLT-1 and the xCT- system within the NAc across the three experimental groups (rats infused with morphine for seven days and treated with vehicle, rats infused with morphine for seven days and treated with secretome and no-morphine control rats). Data are presented as mean $\pm$ SEM, $n=6$ for each experimental group. One-way ANOVA followed by Tukey's post-hoc test.

1016

FIGURE 8



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1018 **Figure 8: Effects of secretome administration on somatic signs of morphine dependence after**
 1019 **morphine deprivation in an animal model of oral voluntary morphine consumption.** Eight week-old
 1020 female Wistar rats voluntarily consumed morphine (~17mg/kg/day) for three weeks. At such time the
 1021 animals received a simultaneous intranasal and intravenous administration of MSC-derived secretome
 1022 (containing 25 µg proteins derived from 1x10⁶ hMSCs) or saline. On the same day, spontaneous
 1023 withdrawal syndrome was induced by removing the morphine bottle. Somatic signs of withdrawal
 1024 syndrome were evaluated 48 hours post-morphine removal during a 30-minute observation period.
 1025 The assessed symptoms included **(A)** weight differences, **(B)** area covered by feces, **(C)** jumps, **(D)**
 1026 ventral/dorsal flexes, **(E)** stretching, **(F)** chewing, **(G)** wet dog shakes, **(H)** forepaw tremors events, and
 1027 **(I)** post-session handling vocalization prevalence. Control group animals had access only to water. Data
 1028 are presented as min to max (n=6-7 per experimental group). *p<0.05; **p<0.01; Tukey's multiple
 1029 comparison tests for normal distribution and non-parametric data evaluated using the Kruskal-Wallis
 1030 test.

FIGURE 9

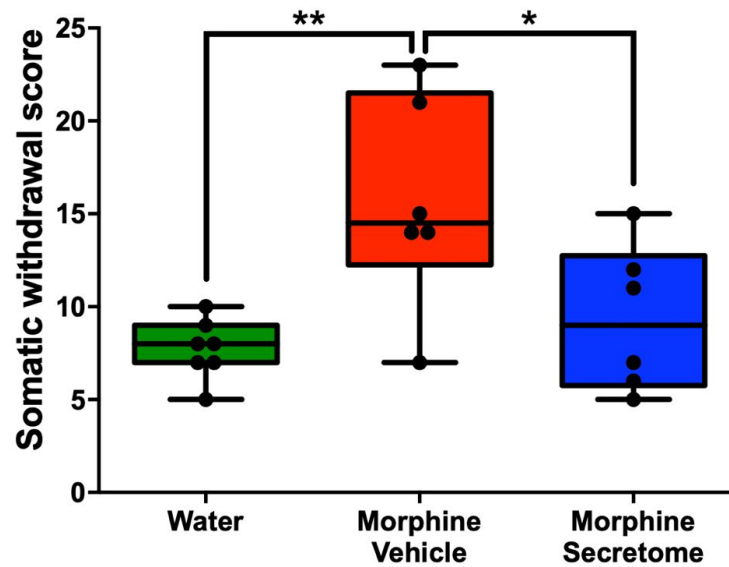
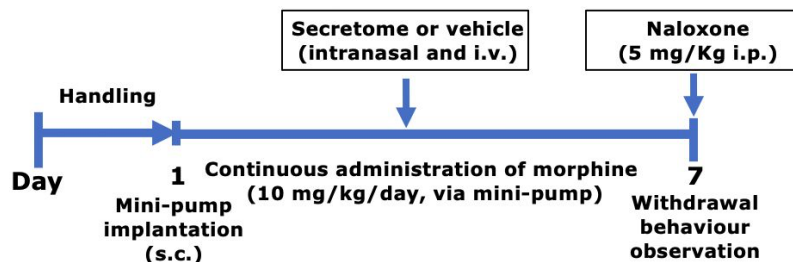
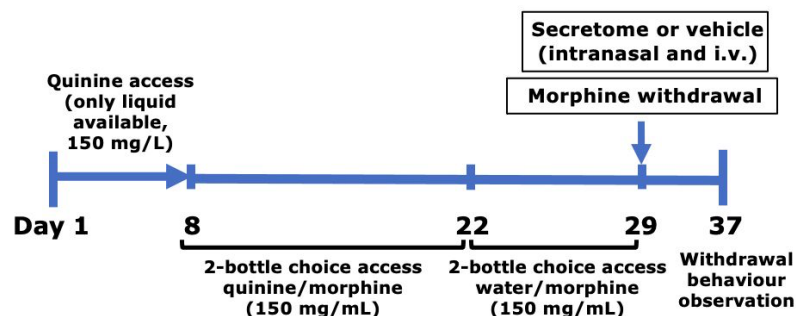


Figure 9: Effects of secretome administration on the combined withdrawal score after morphine deprivation in an animal model of oral voluntary morphine consumption. The somatic withdrawal is depicted using a scoring system based on previous studies (41, 42). Secretome treated rats showed a significant reduction in the somatic withdrawal score compared to vehicle-treated animals. Control group animals had access only to water. Data are presented as min to max $n=6$ in morphine and $n=7$ in the control groups. * $p<0.05$; ** $p<0.01$; Tukey's multiple comparison test for normal distribution.

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SUPPLEMENTARY FIGURE 1

A) Animal model 1: Subcutaneous morphine administration and withdrawal syndrome precipitation by naloxone administration**B) Animal model 2: Oral voluntary morphine consumption and withdrawal syndrome precipitation by morphine abstinence**

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1050 **Supplementary Figure 1: Experimental Design. (A) Animal model 1 Subcutaneous morphine**1051 *administration and withdrawal syndrome precipitation by naloxone administration.* Female Wistar rats

1052 were implanted subcutaneously with osmotic mini-pumps releasing morphine at a dose of 10

1053 mg/kg/day or saline. Three days post pump implantation, the morphine treated animals were

1054 randomly divided into two groups. One group received simultaneously an intranasal (25µg proteins)

1055 and intravenous (25µg proteins) dose of secretome derived from 1×10^6 preconditioned human MSCs,

1056 while the other group received the vehicle. Animals in the saline control group received the intranasal

1057 and intravenous administration of the vehicle. After seven days of continuous morphine exposure, rats

1058 were intraperitoneally injected with 5 mg/kg of the μ -opioid receptor antagonist naloxone to induce1059 withdrawal. **(B) Animal model 2. Oral voluntary morphine consumption and spontaneous withdrawal**1060 *syndrome precipitation by morphine abstinence.* Following an initial seven-day exposure to 0.15

1061 mg/mL quinine hydrochloride as the only fluidic source, the animals transitioned to a two-week
1062 regimen where they were given a choice between two bottles, one contained quinine hydrochloride
1063 (0.15 mg/mL) and the other morphine sulfate (0.15 mg/mL). After this two-week period, the quinine
1064 bottle was removed, leaving the animals with a choice between tap water and morphine sulfate (0.15
1065 mg/mL) for one additional week. A control group that had access only to water was also used.
1066 Withdrawal syndrome was spontaneously induced by discontinuation of morphine access after three
1067 weeks of morphine intake. On the same day, rats were administered simultaneously with an intranasal
1068 and intravenous dose of either secretome or saline. Somatic signs of morphine withdrawal were
1069 recorded for 30 minutes 48 hours later.

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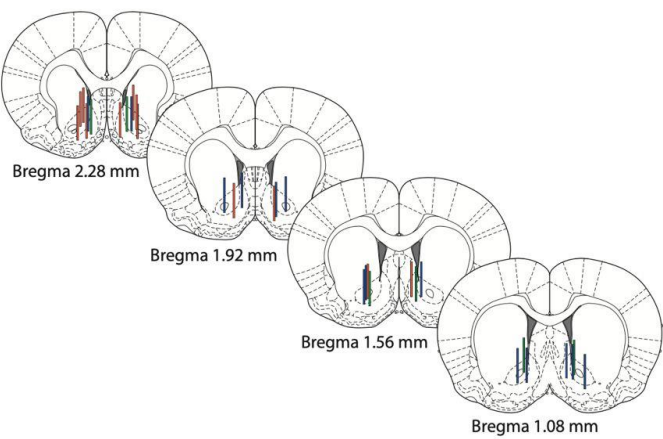
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SUPPLEMENTARY FIGURE 2



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1089 **Supplementary Figure 2: Diagram of coronal sections indicating the placement of microdialysis**
1090 **probes.** Illustrative scheme of the coronal sections showcasing the positioning of the microdialysis
1091 probes. All probe placements coincided with the areas marked by color-coded lines.

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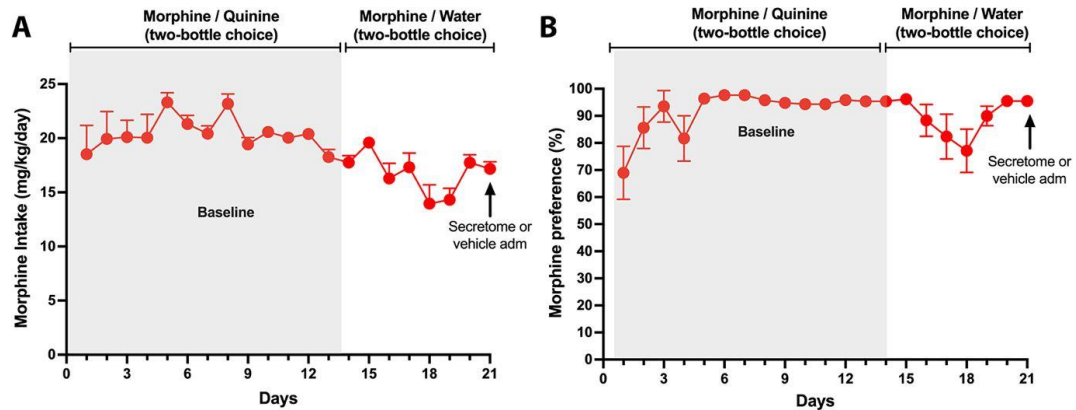
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SUPPLEMENTARY FIGURE 3



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1108 **Supplementary Figure 3: Voluntary morphine intake by Wistar rats.** One week after starting quinine
 1109 intake, animals were exposed to 0.15 mg/mL quinine and 0.15 mg/mL morphine for two weeks using
 1110 the two-bottle choice paradigm. Thereafter, animals were exposed to 0.15 mg/mL morphine and water
 1111 (two-bottle choice) for one additional week. **(A)** Voluntary morphine intake is expressed as mg
 1112 morphine consumed per kg of body weight per day. **(B)** Morphine preference (%). Data are expressed
 1113 as mean $\pm$ SEM. $n = 6$. A control group ($n=7$) was given access to a bottle of water.

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SUPPLEMENTARY TABLE 1

Withdrawal Score		
Behavior	Numbers of events	Score
1.- Stretching	0	0
	1-24	1
	25-49	2
	50-79	3
	80 or more	4
2.- Jumps	0	0
	1-4	1
	5-9	2
	10-19	3
	20-39	4
	40 or more	5
3.- Wet dog shakes	If n > 2	2
4.- Ventral/dorsal flexes	Each n=2	1
5.- Forepaw tremors events	If n > 2	2
6.- Chewing	0	0
	1-4	1
	5-9	2
	10 or more	3
7.- Borrowings	0	0
	1-4	1
	5-9	2
	10-19	3
	20 or more	4
8.- Irritability	Scream when holding	3
9.- Percentage of the area covered by faeces	If % of area > 10	3
10.- Weight difference	—	Not included in the withdrawal score

1126

1127 **Supplementary Table 1: Somatic withdrawal score assessment.** The scoring system is graduated for
 1128 the frequency of each parameter observed during a 30-minute evaluation period in a 5-litre beaker.
 1129 This scoring system was adapted from previous studies (41, 42).

4. Chapter 4

Discussion

As the opioid addiction crisis continues to deepen, the need for robust, effective, and enduring therapeutic interventions has become critically important. This urgent context served as the driving force behind my doctoral research, which focused on investigating the therapeutic potential of human mesenchymal stem cell (hMSC) secretome in mitigating the effects of morphine addiction—one of the most common forms of opioid dependence. The results from two papers generated from this research substantiate the initial hypothesis that hMSC-secretome possesses substantial therapeutic potential in treating morphine addiction and withdrawal syndrome.

In the third paper (considering the publication of the oral morphine model as the first paper), two distinct models were utilized. The first of these mimicked the chronic nature of morphine exposure, an experience common to many human addicts. In this model, a pump was subcutaneously implanted into Wistar rats to administer morphine continuously. The experiment led to a key discovery: the rats treated with hMSC-secretome exhibited a significant reduction in withdrawal symptoms triggered by the opioid antagonist, naloxone. This pivotal finding not only supported our hypothesis, but also underscored the therapeutic role of hMSC-secretome in addiction therapy.

The second animal model was first introduced in the first paper and was further evaluated in the second paper. This model was designed to simulate voluntary oral morphine consumption. Upon administering hMSC-secretome, animals displayed a significant reduction in chronic morphine intake. Importantly, when morphine was reintroduced after a period of forced abstinence, the group treated with the secretome demonstrated a significant decrease in relapse consumption. Indeed, the combined intranasal and intravenous administration of hMSC-secretome was able to reduce chronic self-administration of morphine by 80% to 85%, and after a second dose during a deprivation stage, it further reduced morphine intake during relapse.

Our research findings integrate well within the broader landscape of stem cell research and its potential therapeutic applications in various medical conditions, including addiction. A key facet of our findings was the notable neuroprotective effect of hMSC-secretome, indicated by reduced oxidative stress levels in the hippocampus and nucleus accumbens (third paper). This result signals an important advancement in our understanding of the neurophysiological benefits of hMSC-secretome and augments the scientific discourse around non-cellular therapeutic agents in addiction treatment.

Despite our work focusing on morphine addiction, its implications extend to other substance abuse types. Considering the parallels in neurophysiological processes underlying different substance addictions, the hMSC-secretome presents a plausible therapeutic agent for a range of substance dependence. My doctoral research thus holds the potential for offering

transformative therapeutic options, which could help alleviate the substantial societal and personal burdens associated with opioid addiction.

In summary, our findings validate our initial hypothesis about the therapeutic benefits of hMSC-secretome in opioid addiction, offering a new platform for future exploration. As the journey in addiction science continues, the results from this research raises pertinent questions for future investigations: what are the molecular mechanisms underlying the impact of hMSC-secretome on addiction behavior? Can it be applied to other forms of substance abuse (beyond the already studied)?

The research presented in the third paper introduced additional findings of interest. For instance, continuous morphine administration resulted in reduced extracellular glutamate levels in the nucleus accumbens, an effect not altered by the secretome. However, a significant increase in the slope of no-net-flux glutamate determination induced by secretome treatment suggests potential effects on glutamate elimination dynamics. The lack of changes in the levels of glutamate transporters was unexpected, but these alterations could depend on specific opioid treatment schemes.

Moreover, our studies noted an increase in brain oxidative stress associated with chronic morphine intake, an event linked with morphine consumption relapse. Remarkably, the combined intranasal and intravenous administration of hMSC-secretome significantly reduced both chronic self-administration and relapse to voluntary morphine consumption. These effects were correlated with a reduction in hippocampal oxidative stress.

The compelling findings from this doctoral work underline that hMSC-secretome administration can significantly mitigate morphine withdrawal syndrome, reduce chronic morphine intake and relapse, normalize oxidative stress associated with relapse and chronic consumption, and reduce neuroinflammation during chronic consumption, withdrawal syndrome, and relapse. These outcomes offer immense translational value, particularly considering the alarming rise in opioid consumption and overdose deaths during the COVID-19 pandemic.

Our research journey, while far from complete, has already illuminated critical facets of opioid addiction and potential treatments. Future work should extend this investigation to other relapse evaluation models and further analyze the role of neuroinflammation and oxidative stress. We also need to address whether intravenous administration could prevent withdrawal syndrome induced by potent opioids like fentanyl. These critical questions will undoubtedly shape the direction of future research, bolstering our quest to tackle the global crisis of addiction

5. Conflict of Interest

The researchers confirm that they conducted the study without any commercial or financial ties that may be seen as a possible conflict of interest.

6. Author Contributions

The team consisting of MQ, PBC and FE worked together on the conceptualization, synthesis, and interpretation of the literature. MQ took the lead in drafting the manuscript, which was then critically reviewed by PBC and FE. They collaborated on the final version, and MQ and FE both reviewed and approved the final manuscript.

7. Funding

This work was supported by FONDECYT 1200287 grant to Fernando Ezquer, FONDECYT 3210276 grant to Pablo Berrios, ACT210012 grant to Fernando Ezquer and Paola Morales, the Spanish Ministerio de Ciencias e Innovación PID2019- 109823RB-100/MICINN/AEI/10.13039/501100011033 to Lucia Hipólito and 21212198 ANID Doctorate Grant to Mauricio Quezada

8. Acknowledgements

The authors would like to express their gratitude to Ms Catalina Vallejos and Mr Jorge Ruiz for providing constructive feedback and support.

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