Intranasal delivery of mesenchymal stem cell-derived exosomes reduces oxidative stress and markedly inhibits ethanol consumption and post-deprivation relapse drinking

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Keywords:	GLT1, binge-drinking, stem cell exosomes

Abstract:	Chronic ethanol consumption leads to brain oxidative stress and neuroinflammation, conditions known to potentiate and perpetuate each other. Several studies have shown that neuroinflammation results in increases in chronic ethanol consumption. Recent reports showed that the intra-cerebroventricular administration of mesenchymal stem cells to rats consuming alcohol chronically markedly inhibited oxidative-stress, abolished neuroinflammation and greatly reduced chronic alcohol intake and post deprivation relapse-like alcohol intake. However, the intra-cerebroventricular administration of living cells is not suitable as a treatment of a chronic condition. The present study aimed at inhibiting ethanol intake by the noninvasive intranasal administration of human mesenchymal stem cell products: exosomes; microvesicles (40 to 150 nm) with marked antioxidant activity extruded from mesenchymal stem cells. The exosome membrane can fuse with the plasma membrane of cells in different tissues, thus delivering their content intracellularly. The study showed that the weekly intranasal administration of mesenchymal stem cell-derived exosomes to rats consuming alcohol chronically (i) inhibited their ethanol intake by 80% and blunted the relapse-like "binge"

drinking that follows an alcohol deprivation period and ethanol re-access. (ii) Intranasally administered exosomes were found in the brain within 24-hours; (iii) fully reversed both alcohol-induced hippocampal oxidative-stress, evidenced by a lower ratio of oxidized to reduced glutathione, and neuroinflammation, shown by a reduced astrocyte activation and microglial density and (iv) increased glutamate transporter GLT1 expression in nucleus accumbens, counteracting the inhibition of glutamate transporter activity, reportedly depressed under oxidative-stress conditions. Possible translational implications are envisaged.

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Running title: Exosomes and ethanol intake

Keywords: ADE, GLT-1; noninvasive, binge-drinking, mesenchymal stem cells, exosomes

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ABSTRACT

Chronic ethanol consumption leads to brain oxidative stress and neuroinflammation, conditions known to potentiate and perpetuate each other. Several studies have shown that neuroinflammation results in increases in chronic ethanol consumption. Recent reports showed that the intra-cerebroventricular administration of mesenchymal stem cells to rats consuming alcohol chronically markedly inhibited oxidative-stress, abolished neuroinflammation and greatly reduced chronic alcohol intake and post deprivation relapse-like alcohol intake. However, the intra-cerebroventricular administration of living cells is not suitable as a treatment of a chronic condition. The present study aimed at inhibiting ethanol intake by the noninvasive intranasal administration of human mesenchymal stem cell products: exosomes; microvesicles (40 to 150 nm) with marked antioxidant activity extruded from mesenchymal stem cells. The exosome membrane can fuse with the plasma membrane of cells in different tissues, thus delivering their content intracellularly. The study showed that the weekly intranasal administration of mesenchymal stem cell-derived exosomes to rats consuming alcohol chronically (i) inhibited their ethanol intake by 80% and blunted the relapse-like "binge" drinking that follows an alcohol deprivation period and ethanol re-access. (ii) Intranasally administered exosomes were found in the brain within 24-hours; (iii) fully reversed both alcohol-induced hippocampal oxidative-stress, evidenced by a lower ratio of oxidized to reduced glutathione, and neuroinflammation, shown by a reduced astrocyte activation and microglial density and (iv) increased glutamate transporter GLT1 expression in nucleus accumbens, counteracting the inhibition of glutamate transporter activity, reportedly depressed under oxidative-stress conditions. Possible translational implications are envisaged.

INTRODUCTION

Chronic alcohol intake is the most common cause of peripheral and central nervous system toxicity (Pfefferbaum *et al.* 1998). Studies in humans and rodents have shown that chronic alcohol consumption leads to an increase of inflammatory cytokines, both in the brain (Crews and Vetreno, 2016; Montesinos *et al.* 2016; Schneider *et al.* 2017) and the periphery (Leclercq *et al.* 2012). Pro-inflammatory conditions and the administration of lipopolysaccharide (LPS) have been shown to also increase voluntary alcohol consumption in animals (Blednov *et al.* 2012; Mayfield *et al.* 2013). In alcoholics, the levels of pro-inflammatory cytokines in blood correlate positively with alcohol craving (Leclercq *et al.* 2012). Ethanol intake, via the generation of acetaldehyde, weakens the tight junction intestinal barrier and promotes LPS influx from the gut into the portal circulation (Ferrier *et al.* 2006), leading to increases in systemic TNF-α and induction of neuroinflammation via a brain TNF-α receptor (Qin *et al.* 2007).

Recent studies show that inflammation and oxidative stress are linked via the mitochondrial uncoupling action and oxygen radical generation induced by TNF-α (Kastl *et al.* 2014). In turn, reactive oxygen species (ROS) are known to inactivate the NF-kB inhibitor IkB, leading to the generation of pro-inflammatory cytokines including TNF-α (Canty *et al.* 1999). Thus, a vicious cycle exists between inflammation and oxidative stress (Crowley 2014). Which of these precedes the other is not clear. Increases in brain ROS have been reported following chronic ethanol intake in rodents (Costa *et al.* 2015; Montoliu *et al.* 1994), likely via increases in cytochrome P450 (CYP)2E1 (Montoliu *et al.* 1994). In addition to the oxidative stress

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generation by CYP2E1, in dopaminergic terminals the presynaptic re-uptake of dopamine

generates hydrogen peroxide (and oxygen radicals) by the action of monoamine oxidase on

dopamine (Cunha-Oliveira *et al.* 2013). Further, dopamine released into a milieu with a

higher pH than the low pH in dopamine vesicles leads to catechol auto-oxidation into

semiquinone and quinone, with the intermediate generation of superoxide ion (Hastings,

2009). Marked increases in the GSSG/GSH ratio, an indicator of cell oxidative stress, have

also been shown in the brain of rats fed alcohol chronically (Ezquer *et a*l. 2017).

Acetaldehyde generated from the brain metabolism of ethanol is required to initiate ethanol

intake via the release of dopamine into the nucleus accumbens, but acetaldehyde *does not*

perpetuate chronic alcohol intake once a steady state intake is attained (Israel *et al.* 2017b).

Intake perpetuation and relapse for drugs of abuse occurs mostly by cued-activation of

nucleus accumbens via the hippocampus (Scofield *et al,* 2016; Fotros *et al*. 2013). A major

mechanism of chronic ethanol intake and of ethanol cued-relapse results from an elevation

of extracellular glutamate in nucleus accumbens (Gass *et al*. 2011). In this area, the level of

astrocyte glutamate transporter GLT1, which keeps extracellular glutamate levels within the

physiological range, is strongly associated with ethanol volition. An elevation of GLT1 by the

systemic administration of the GLT1-inducer ceftriaxone markedly inhibits chronic alcohol

intake (Das *et al*. 2015; Rao *et al*. 2015). Conversely, a high ethanol consumption induced by

chronic ethanol intake is associated to a reduced activity of the GLT1 transporter. As

indicated above, chronic ethanol intake markedly increases the levels of ROS, known to

oxidize and cross-link cysteine residues in GLT1, which greatly inhibit the activity of this transporter without altering GLT1 protein levels (Trotti et al. 1998).

Mesenchymal stem cells (MSCs) are emerging as a clinical option in the treatment of diseases in which dysregulation of the immune system is involved (Wang et al. 2016). The immunomodulatory potential of MSCs, due to a number of mechanisms for various targets of the immune system, is well documented (Gebler et al. 2012) and includes the release of the anti-inflammatory molecules IL-5, IL-10 and IDO, among other (Ezquer et al. 2017). Due to the concerted secretion of several anti-inflammatory modulators, mesenchymal stem cells have been termed the "guardians of inflammation" (Prockop and Oh 2012). Mesenchymal stem cells also share the ability to withstand severe oxidative stress, due to the expression of high levels of antioxidant systems (Valle-Prieto and Conget 2010); these anti-inflammatory and anti-oxidative properties are greatly improved by incubating the MSCs with pro-inflammatory cytokines (Ezquer et al. 2017). Antioxidants released upon MSC activation were shown to markedly protect against ROS-induced cell damage in several pathological models (Kadekar et al. 2016).

Recent in vivo studies have shown that the intra-cerebroventricular administration to chronically alcohol ingesting rats of bone marrow-derived and adipose tissue-derived rat MSCs (Israel et al. 2017a) or adipose tissue-derived human MSCs (Ezquer et al. 2017) markedly reduce chronic alcohol intake and inhibit relapse-like drinking. The single administration of activated human MSCs inhibited ethanol intake for 3 to 5 weeks. While

these "proof of principle" studies support the hypothesis that neuroinflammatory-oxidative

stress system can modify chronic ethanol intake and relapse drinking, the intra-

cerebroventricular administration of living cells is not readily applicable as a treatment of a

chronic disease such as alcoholism. Thus, non-invasive alternatives must be sought to

replace MSCs per se.

A number of studies have shown that MSCs produce exosomes; extracellular vesicles that

are released from cells upon fusion of an intermediate endocytic compartment, the

multivesicular body (MVB), with the plasma membrane (Koniusz *et al*. 2016). Once released,

exosomes are able to fuse with the plasma membrane of cells in different tissues, thus

delivering their content intracellularly. It has been proposed that exosomes constitute an

important mechanism contributing to the therapeutic effects of MSCs (Jing et al. 2018;

Phinney and Pittenger, 2017). Recent studies have shown the potential of mesenchymal

stem cell-derived exosomes to reduce oxidative stress via peroxiredoxins and glutathione S-

transferase present in them (Arslan et al. 2013), suggesting that exosomes could be used to

reduce oxidative stress in the brain of alcoholic animals. Additional studies both *in vitro* and

in vivo have shown the marked ability of MSC-derived exosomes to reduce oxidative stress

and cell damage in several pathological conditions including hydrogen peroxide incubation,

carbon tetrachloride administration, acetaminophen tissue injury, and ischemic

cardiomyopathy (Damania *et al*. 2018; Shi *et al*. 2018).

Additionally, the small size of exosomes (40-150 nm) allows their non-invasive intranasal administration. In this study, we determined whether the intranasal administration of exosomes derived from activated human MSCs to high-alcohol drinker rats (a) reaches the brain, (b) reduces ethanol-induced neuroinflammation and brain oxidative stress, (c) increases the levels of the glutamate transporter GLT1 and (d) inhibits chronic alcohol intake and relapse-like drinking.

MATERIALS AND METHODS

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

stem cells (AD-MSCs)

Human AD-MSCs were isolated from fresh subcutaneous adipose tissue samples (abdominal

region) obtained, after written informed consent, from aspirates of four patients undergoing

cosmetic liposuction at Clínica Alemana, Santiago, Chile. All protocols were approved by the

Ethics Committee of Facultad de Medicina, Clínica Alemana-Universidad del Desarrollo.

Adipose tissue-derived MSCs were isolated and expanded as previously described (Ezquer *et*

al. 2017; Oses *et al*. 2017). After two subcultures, cells were characterized according to their

osteogenic and adipogenic differentiation potential; by the presence of putative human MSC

markers (CD13, CD29, CD73, CD90 and CD105) and by the absence of markers characteristics

of other cell lineages (CD31, CD45 and CD235a) as previously described (Ezquer *et al*. 2017;

Oses *et al*. 2017).

Activation of human AD-MSCs

Human AD-MSCs (passage 3) at 70% of confluence were activated by incubation in alpha-

MEM (Gibco, Grand Island, NY) supplemented with 10% exosome depleted fetal bovine

serum (Gibco), 10 ng/ml TNF-α and 15 ng/ml IFN-γ (R&D Systems, Minneapolis, MN) for 48

hours as previously described (Ezquer *et al*. 2017). Preliminary studies showed that, unlike

effect of exosomes derived from activated hMSC (vide infra), exosomes generated by non-

activated hMSC had minimal or no effects in reducing chronic alcohol consumption or in

changing ethanol intake in the relapse-like condition.

Collection and characterization of MSC-derived exosomes

Forty-eight hours after MSC activation, secretomes were obtained by harvesting the culture media. The media was centrifuged at 400g for 10 minutes to remove whole cells. Supernatants were recovered, centrifuged again at 5,000g and filtered in 0.22 μm syringe filters to remove cell debris. For exosome isolation, the media were ultracentrifuged at 170,000g for 1 hour to pellet exosomes. The pellets were washed with PBS and ultracentrifuged again as previously described (Cheng *et al*. 2017). Exosome pellets were diluted in filtered saline and exosome protein concentration was determined by a MicroBCA Protein Assay kit (Thermo Scientific, Rockford, IL). The size and concentration of exosomes were analyzed by NanoTracking System Analysis (NTA) using the NanoSight NS500 equipment (NanoSight Ltd, Amesbury, United Kingdom) and by transmission electron microscopy with a Phillips Morgagni electron microscope as previously reported (Damania *et al*. 2018). Gel electrophoresis and Western blots were performed to confirm the enrichment of exosome markers (GM130, ALIX, Flotilin and EpCAM) in exosome samples compared to whole cell lysate using the Exosomal Marker Ab sampler kit (Cell Signaling Technology, Danvers, MA). Calnexin and HSP60 markers were used to discard endoplasmic reticulum contamination.

Animal model of chronic alcohol consumption

Two-month-old female Wistar-derived rats selectively bred as alcohol consumers (University of Chile Bibulous; UChB) (Quintanilla *et al*. 2006) were used in the experiments. Animals are regularly fed a soy-protein rodent diet (Cisternas, Santiago Chile). Animal experimental

procedures were approved by the Ethics Committee for Studies with Laboratory Animals at the Faculty of Medicine, University of Chile. Animals weighing 180 to 250 g were housed in individual cages at a temperature- and humidity-controlled room under 12-hour light/dark cycle for one week to acclimate animals to the testing conditions. During this time food and water were freely available.

For induction of chronic alcohol intake, animals had continuous 24-hours free choice access to 10% v/v ethanol and water for 14 weeks. On the last two weeks' animals could choose between 10% ethanol, 20% ethanol and water from separate tubes. Relapse-like alcohol drinking was assessed after the animals were deprived of ethanol for 14 days and then allowed re-access to 10% and 20% ethanol solutions for only 60 minutes as previously described (Ezquer *et al*. 2017).

Administration of MSC-derived exosomes directly into the brain.

Prior to determining the effect of exosomes administered intranasally, the effect of exosomes was tested by the direct administration of exosomes into the brain cerebrospinal fluid. After approximately 100 days of chronic alcohol intake, rats were pre-anesthetized with chloral hydrate (280 mg/kg i.p.) and mounted on a David Kopf stereotaxic frame with the skull oriented according to the Paxinos & Watson (1998) rat brain atlas. Anesthesia was kept with a mixture of air and isoflurane along the full procedure. The skull was exposed and a hole drilled for implanting the tip of a 10μl Hamilton syringe into the left lateral ventricle (A -0.8; L -1.6; V -3.4), the ventral coordinate calculated from the surface of the brain. Ten microliters of saline containing 1.5×10^9 exosome particles (7.5 μg of exosome proteins;

derived from $1.0x10^6$ MSCs) were slowly injected. After the ICV injection, the syringe was removed and the wound sutured, adding an antiseptic solution. Control animals were ICV injected with 10 µl of saline –the standard control in this field.

Intranasal administration of MSC-derived exosomes

In separate studies, after approximately 100 days of chronic alcohol intake, rats were anesthetized with chloral hydrate (280 mg/kg i.p.) and placed in a supine position. Twenty microliters of exosome solution were administered intranasally as drops delivered from a small pipette every 5 minutes (four times in each nostril) into alternative sides of the nasal cavity for a total of 20 minutes. A total volume of 160 µl of saline containing 1.5×10^9 exosome particles (7.5 µg of exosome proteins; derived from $1.0x10^6$ MSCs) was delivered into the nasal cavity. Intranasal administration was repeated once a week for five weeks. Control animals received 160 µl of saline by the above schedule (vide infra). Overall, the method used for intranasal instillation was adapted from Long *et al.* (2017). However, unlike the studies of Long *et al* in mice, intranasal hyaluronidase pretreatment was not used since preliminary experiments with exosomes did not show differences in exosome delivery to the rat brain with or without hyaluronidase.

Determination of chronic daily ethanol intake, ethanol intake during the alcohol deprivation effect (ADE) condition, neuroinflammation and GSSG/GSH ratio following intranasal exosome administration.

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To determine the effect of exosome treatment on chronic ethanol intake and on the alcohol deprivation effect (ADE), two groups of rats (n= 5-6 rats per group) that had consumed ethanol for ninety-one days were used. One group received at weekly intervals 5 intranasal doses of exosomes of 160 μl (containing 7.5 micrograms divided into two aliquots; one for each nostril); the second group received 160 μl of saline. The first three doses were administered while the rats were under chronic ethanol intake, and the last two doses were administered while the animals were under a 17-day period of ethanol deprivation. Re-access to 10% and 20% ethanol for 60-minute period was allowed three days after the last intranasal exosomes administration (Ezquer et al. 2017). Immediately after the 60-minute re-access, blood samples were obtained for ethanol levels determination. Thereafter, animals were euthanized and brain samples were obtained for the determination of hippocampus GSSG/GSH ratio and astrocytic and microglial reactivity. It is noted that (vehicle control) animals that had consumed alcohol chronically and were deprived of ethanol for 2 weeks consumed 1.6 g ethanol/kg in 60 minutes. Their chronic ethanol intake prior to the deprivation is of the order of 12 g ethanol/24 hours; equivalent to 0.5 g ethanol/60 minutes. Thus, following the alcohol deprivation the animals consume 3-times more ethanol/ 60 minutes than they do upon the chronic intake period.

A second group of rats that had ingested ethanol for ninety-one days received only one intranasal dose of exosomes or vehicle and after 48 hr animals were euthanized to obtain brain samples for determination of hippocampus GSSG/GSH ratio and determination of

mRNA levels of glutamate transporters in nucleus accumbens and prefrontal cortex. Ethanol

intake was expressed as g of ethanol consumed/kg body weight/60 minutes.

Ethanol intake and water intake were determined every 24 hours from volume difference of

100 ml inverted graduated cylinders. Ethanol intake was expressed as g of ethanol

consumed/kg body weight/day. Water intake was expressed as ml of water consumed/kg

body weight/day.

Blood ethanol determination

After rats had 60 minutes of re-access to ethanol and water, 100 µl of blood samples was

collected without anticoagulant from the tip of the tail under moderate sedation with

acepromazine (1 mg/kg i.p.). Blood samples were immediately added to 0.9 ml of distilled

water in a glass vial sealed with Mininert valves (Supelco, Bellefonte, PA). Samples were

analyzed by headspace gas chromatography (Perkin Elmer SRI 8610) as previously described

(Quintanilla *et al*. 2007).

Determination of GSSG/GSH ratio in hippocampus following a single intranasal exosome
administration.

These studies, aimed at determining the relationship between exosome effects on ethanol

intake and neurochemical changes, were conducted 48-hours following *a single* intranasal

exosome administration. After the intranasal administration of exosomes or saline, rats

were anesthetized with chloral hydrate (280 mg/kg i.p.) and perfused intracardially with 100

ml of warm PBS (pH 7.4).   Hippocampi were extracted and mixed with three volumes of ice-

cold potassium buffer containing 5 mM EDTA, pH 7.4. Reduced glutathione (GSH) and

glutathione disulfide (GSSG) contents were determined as previously described (Perez-Lobos

et al. 2017). This method for GSSG and GSH determination was used throughout the study.

Quantification of mRNA levels of glutamate transporters in nucleus accumbens and

prefrontal cortex following intranasal exosome administration

Forty-eight hours after a single intranasal administration of exosomes or saline, total RNA

from nucleus accumbens and prefrontal cortex was purified using Trizol (Invitrogen, Grand

island, NY). 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 to amplify the glutamate transporters GLT1, GLT1a, GLT1b, xCT and GLAST using

a Light-Cycler 1.5 thermocycler (Roche, Indianapolis, IN). To ensure that amplicons were

generated from mRNA and not from genomic DNA, controls without reverse transcriptase

during the reverse transcription reaction were included. Relative quantifications were

performed by the ΔΔCT method. The mRNA level of each target gene was normalized

against the mRNA levels of the housekeeping genes actin and glyceraldehyde-3-phosphate

dehydrogenase (GAPDH) in the same sample.

Evaluation of exosome distribution in the brain after intranasal administration

To determine the ability of intranasally administered exosomes to reach the brain, exosomes

were stained with PKH26 membrane dye (Sigma-Aldrich, St. Luis, MO) according the

manufacturer`s instructions. Stained exosomes were washed in 4 ml of saline and

concentrated again by ultracentrifugation. One hundred and sixty microliters of saline

containing 1.5×10^9 labeled exosome particles or the same volume of PKH26-saline control

were intranasally administered as described above. Two, 6, 12 and 24 hours after exosome

administration, animals were anesthetized and intracardially perfused with 100 ml of 0.1M

warm PBS (pH 7.4), followed by 200 ml of formalin solution. The brains were carefully

removed and PKH26 fluorescence was evaluated in dorsal, ventral and sagittal views of the

brains using an In-Vivo MS FX PRO Imaging System (Bruker).   The brains were exposed to the

white light for 0.175 sec and then to 550 nm excitation wavelength for 10 sec, capturing the

fluorescence at 600 nm with the highest resolution. Auto-fluorescence was corrected for

each brain and view respectively to the saline control auto-fluorescence. Subsequently, a

merge of both images was created.

Histochemistry for PKH26

Para-sagittal sections of the brain (30 μm thick) were obtained using a cryostat (Thermo

Scientific Microm HM 525 Cryostat). The sections were mounted on silanized slides and

stored pending further experiment.   Sections were washed with 0.1 M PBS and incubated

with 4,6 diamino-2-phenylindol (DAPI; Invitrogen 0.02 M; 0.0125 mg/mL) for 2 h. After

rinsed, the samples were mounted with Fluoromount, and examined by confocal microscopy

(Olympus-fv10i).

Determination of astrocyte and microglial reactivity.

Immunofluorescence against the astrocyte marker glial fibrillary acidic protein (GFAP) and

ionized calcium binding adaptor molecule 1 (Iba-1) a marker for macrophage/microglia, were

evaluated in coronal sections of the hippocampus (30 μm thick) as previously reported

(Ezquer *et al.* 2018). Briefly, coronal sections were washed with 0.1 M PBS incubated with

blocking solution (0.3% Triton X-100, 0.1% BSA, and 10% NGS in PBS) for 1h and then

incubated with a primary rabbit monoclonal anti-IBA-1 antibody (cat #019-19741, Wako

Pure Chemical Industries, Chuo-Ku, Osaka, Japan, at a 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 488 (Thermo Fisher

Scientific, Whaltam MA, 1:500 in 0.3%Triton X-100, 1%BSA, 1% NGS) for 1 h in the dark at

room temperature. After that, sections were rinsed, incubated in blocking solution (5%

normal goat serum 1% BSA and 0.3% Triton X-100 in PBS) for 1h and subsequently with

primary antibody against GFAP (G3893, Sigma-Aldrich, at a 1:500 dilution in blocking

solution) overnight at 4°C in darkness. Samples were rinsed with PBS, incubated with goat

anti-mouse secondary antibody (Alexa Fluor 594, Thermo Fisher Scientific, at a 1:500 dilution

in 0.3%Triton X-100, 1%BSA, 1% NGS) and (DAPI, Invitrogen 0.02 M; 0.0125 mg/ml; for

nuclear labeling) for 2 h. After rinsing, the samples were mounted with Fluoromount and

examined by confocal microscopy (Olympus-fv10i). Nine Microphotographs per animal

(three areas per slice) were taken from the *stratum radiatum* of the hippocampus (between

Bregma -3.14 – -4.16mm, Paxinos & Watson (1998)). The area analyzed for each stack was

0.04 mm^2 and the thickness (Z axis) was measured for each case. The total length and

thickness of astrocyte primary processes was assessed for six GFAP positive cells per Z-stack,

using FIJI image analysis software (http://fiji.sc/Fiji) as previously reported (Ezquer *et al*.

2018).

The microglial cell density was estimated according to Morales *et al*. 2008 in the same nine

microphotographs per animal used for the astrocyte reactivity determination. The Iba-

1/DAPI positive cells were counted when immunoreactivity overlapped at least three levels

through a section (Z-step 1 μm) using FIJI image analysis software. Microglial cell density was

estimated as the average of Iba-1/DAPI positive cells divided by the area (X,Y axis) of the

stack and the thickness (Z axis) of the slice, and expressed as cells/mm^3.

Statistical Analyses

Statistical analyses were performed using Graph Pad Prism software (San Diego, CA). Data

are expressed as means ± SEM. Two-way (treatment x day) analysis of variance (ANOVA),

followed by Bonferroni`s post hoc test or ANOVA for repeated measures when required, was

used to compared the ethanol or water intake between vehicle and exosome groups. One-

way ANOVA followed by Tukey`s post hoc test was used to analyze the data on GSSG/GSH

ratios and mRNA levels of glutamate transporters. One-way ANOVA followed by Bonferroni

post hoc test was used to analyze the data on astrocyte reactivity and microglial number.

When appropriate, a Student`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.

RESULTS

Characterization of exosomes derived from activated human adipose tissue-derived mesenchymal stem cells (AD-MSCs)

Figure 1A shows the size distribution of extracellular vesicles (exosomes) was determined by the NanoTracking Analysis system. The major peaks indicate that the vesicles range between 70 to 200 nm, with the major concentration in the range of 100 to 120 nm, the size previously described for exosomes. Figure 1B shows exosomes of the order of 80 to 100 nm visualized by transmission electron microscopy. Note the electron darker area surrounding the exosomes, indicative of membrane-associated proteins. Figure 1C depicts the Western blot showing the classic exosome markers GM130, ALIX, Flotilin and EpCAM, compared with the whole MSCs lysate. Absence of calnexin and HSP60 markers in the exosome fraction discarded endoplasmic reticulum contamination in the exosome samples.

Intra-cerebroventricular (ICV) administration of exosomes derived from activated human AD-MSCs markedly inhibits chronic ethanol intake

Figure 2 shows that ICV exosome administration (1.5×10^9 exosome particles) markedly inhibits chronic ethanol intake [$F_{treatment(1,528)}$: 667.3; $p < 0.001$] mimicking the rapid inhibition of ethanol intake and the extended action previously reported upon the administration of whole MSCs (Ezquer *et al*. 2017), which are expected to release their exosomes *in vivo*. *Post hoc* test revealed that the single ICV injection of exosome particles induced a significantly inhibition of ethanol intake that lasted 38 days. Noteworthy, most of the inhibitory effect on ethanol intake of ICV administered exosomes was seen within the first 24-hours.

Intranasal administration of exosomes derived from activated human AD-MSCs inhibits chronic ethanol intake and reduces the relapse-like drinking after deprivation

Figure 3A shows the inhibitory effect of exosomes administered by the intranasal route. Once again, the inhibitory effect was very fast. Two-way ANOVA of ethanol intake indicates a significant effect of the dose of 1.5 x10^9 exosomes administered intranasally once a week for 3 weeks compared with the group treated with vehicle [$F_{treatment(1,192)}$.924.6; p <0.001]. *Post hoc* test revealed that rats treated with the intranasal dose of 1.5 x10^9 exosomes administered once a week for 3 weeks displayed a significantly inhibition of ethanol intake during all days recorded versus vehicle treated rats**.** A single dose of 1.5 x10^9 exosomes inhibited ethanol intake by 61% within 48 hours. One half of this effect was lost in 5 days. The second intranasal exosome administration 7 days after the first administration lowered alcohol intake to levels that were 75% below control levels. Compared with the ethanol intake resulting from the first intranasal dose, the second intranasal dose induced a significantly lower ethanol intake [F(1,13):26.89; p <0.001]. The third weekly intranasal administration of exosomes inhibited ethanol intake by 84% versus that of control groups, while not significantly different from that after the second exosome dose. The exosome-induced inhibition of intake of alcohol solutions were counter-balanced by increases in water intake (Figure 3B ($F_{treatment(1,192)}$: 283.1; p <0.0001), thus indicating that animals keep their water homeostasis. Four days after the third exosome administration, animals were deprived from ethanol while a fourth and fifth exosome administration were continued on the same weekly schedule. Three days after the last intranasal administration of exosomes, animals were offered 10% and 20% ethanol solutions (water tube also available) for only 60-

minutes. Animals in the control group ingested 1.65 ± 0.09 g ethanol/60 minutes, while exosome treated animals ingested a significantly lower amount of 0.75 ± 0.24 g ethanol/60 minutes (t-test= 3.355; $p<0.01$; n: 6 rats/group) (Figure 3C). Blood ethanol determined immediately after the 60-minute ethanol re-access was 115 ± 20 mg/dl for control animals (which in humans would be considered binge-drinking) while in exosome treated animals blood ethanol levels reached 58.6 ± 14 mg/dl (t-test= 2.318; $p<0.049$; n: 6 rats/group ($p<0.05$). Animals were euthanized immediately after the 60-minute ethanol re-access and their hippocampi were processed for astrocyte and microglial immunofluorescence, as described in Methods. Marked astrocytosis (increase in length and thickness of astrocytic processes) was observed in animals that had ingested ethanol chronically (Figure 4A top mid). The intranasal administration of MSC-exosomes reduced the increase in the length of astrocytic processes (one-way ANOVA; $F_{(2,2527)} = 188,4$ $p < 0.0001$) (Figure 4A top right and 4B) and thickness of primary astrocytic processes (one-way ANOVA; $F_{(2, 1277)} = 33,93$; $p < 0.0001$) (Figure 4A top right and 4C) in the hippocampus observed in ethanol-vehicle group compared with the water group.

Animals exposed to ethanol also showed significant increases in microglial numbers (Figure 4A middle, arrows), indicative of neuroinflammation (see Chastain and Sarkar 2014), which did not proceed to an ameboid-like microglial character. Exosome administration fully reversed the increases in ethanol-induced microglial number (one-way ANOVA; $F_{(2, 58)} = 6,66$; $p < 0.001$) (Figure 4A lower right, arrows; Fig 4D). A minor change in microglial activation is in line with studies in alcohol dependent patients (Hillmer et al 2017).

Exosome administration also led to the normalization of (GSSG/GSH) oxidative stress (Figure

5), [one-way ANOVA; $F_{(2,12)}$ = 5.17, p < 0.01] indicating that MSC-exosomes administered via

the intranasal route are able to reduce the ethanol-induced neuroinflammation and

oxidative stress.

**A single intranasal administration of exosomes derived from activated human AD-MSCs
reduces hippocampal oxidative stress and increases glutamate transporters expression in
nucleus accumbens.**

Studies conducted on a separate group of animals (Figure 6A), confirm that marked

inhibition of chronic ethanol intake (60 to 70%; p<0.001) is observed 48 hours after a single

intranasal administration of exosomes.   Animals were sacrificed at such time and the ratio

of oxidized to reduced glutathione (GSSG/GSH) was determined in hippocampal tissue. As

shown in Figure 6B, the GSSG/GSH levels of animals that had ingested alcohol chronically

and intranasally administered the vehicle were 300% those of naïve animals ingesting only

water, an effect that was fully abolished within 48 hours of the intranasal administration of

exosomes, thus indicating that the marked oxidative stress induced by chronic ethanol

intake is suppressed by the MSC-generated exosomes [$F_{(2,12)}$:15.13; p <0.0001]. In these

animals, the expression of the glutamate transporter carriers GLT1 and xCT was determined

in nucleus accumbens and prefrontal cortex. These brain areas were chosen in accordance

with the studies of Rao *et al*. (2015), who indicated that the activator ceftriaxone showed its

full effect in inhibiting ethanol intake and elevating accumbens and prefrontal cortex GLT1

levels within 24 to 48 hours. Figure 7 shows that the total or combined GLT1 gene expression

in nucleus accumbens (comprising the expression of GLT1a and GLT1b genes), was

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significantly increased versus chronic ethanol vehicle-treated controls and water consuming

controls ($F_{(2,12)}$: 11.63; p <0.0016) following a single intranasal exosome administration. In

line with recent studies (LaCrosse *et al*. 2017; Rao *et al.* 2015) showing a coordinated

increase of both the Na-glutamate GLT1 transporter and the cysteine-glutamate xCT

transporter, the latter was also significantly elevated 48-hours after the single intranasal

exosome administration (Figure 7). No changes were observed in the expression of

glutamate-aspartate transporter (GLAST). Noteworthy, the changes in GLT1 and xCT

transporters induced by exosome administration were only observed in nucleus accumbens

and not in prefrontal cortex (Figure 7) (see Discussion).

Intranasal administration of exosomes derived from activated human AD-MSCs efficiently

reaches the brain

As shown in Figure 3A above, a clear inhibition (60 to 70%) of ethanol intake is observed

within the first 24 hours of intranasal exosome administration. In line with this observation,

Figure 8 A shows that fluorescence from exosomes (visualized by the fluorescent tag PKH26)

was clearly observed in the brain at 6, 12 and 24 hours after their intranasal administration.

Figure 8B shows that PKH26 positive exosomes (red fluorescence) were found in the cell

cytoplasm (white arrow), with a reticular distribution, suggesting cellular exosomes uptake

by neurons (fluorescent exosome uptake also observed in glial and endothelial cells is not

shown). PKH26 positive exosomes can also be seen in the extracellular space. These results

can be observed in olfactory bulb, cerebral cortex, dorsal and ventral striatum, thalamus,

hypothalamus and brainstem.

DISCUSSION

The main finding in this study is the demonstration that the intranasal administration of mesenchymal stem cell-derived exosomes reach the brain and markedly inhibits ethanol intake; suggesting a possible translational value. To initially assess the effect of exosomes on chronic ethanol intake, a single dose of exosomes was injected intra-cerebroventricularly. The exosomes inhibited ethanol intake by 60 to 70% in 24 to 48 hours. The inhibitory effect was prolonged since half of the inhibition lasted 21-28 days. Subsequent studies showed that the intranasal administration of the same dose of exosomes inhibited chronic ethanol intake by 60% within the first 48 hours, with a significant inhibition (45%; p<0,001) observed at 24 hours, in line with the presence of fluorescence from labelled exosomes in the brain at that time. Such a fast effect, in the absence of a specific localization of exosomes in discrete brain areas suggests a paracrine effect. Following three intranasal administrations of exosomes on a weekly schedule, the inhibition of chronic ethanol intake reached 75 to 80%. The intranasal administration of exosomes also inhibited alcohol relapse by 55% following ethanol deprivation (ADE), preventing the increases of blood ethanol levels that in humans (>80 mg/dl) would be considered "binge-drinking".

In studies of many drugs of abuse, associated cues trigger a relapse-self-administration (Li *et al*. 2015). Studies by (Gass *et al*. 2011) showed in rats that cues associated with previous alcohol self-administration (lights, tone) markedly elevate extracellular glutamate in nucleus accumbens. In humans with alcohol-use-disorders, oxygen consumption is increased in nucleus accumbens upon presentation of alcohol-related olfactory cues (Kareken *et al*.

2004). In line with these findings, the incentive-salience generated upon the activation of nucleus accumbens enhances an associative learning of the reward context (Juarez and Han, 2016). It is noted that the mechanism that initiates ethanol intake in rats; namely the generation of acetaldehyde in the ventral tegmental area, no longer applies in animals that have ingested alcohol chronically (Israel *et al*. 2017b; Peana *et al*. 2015). Thus, ethanol odor might be the cue recognized by chronically ethanol consuming rats.

Present studies also demonstrate that chronic alcohol intake leads to marked oxidative stress, as shown by increases in the GSSG/GSH ratio. Trotti and coworkers (1998), have shown that GLT1 activity is inhibited under oxidative stress conditions, which is expected to elevate extracellular glutamate levels. The administration of exosomes led to an increased GLT1 expression (mRNA) and, consistent with a reciprocal interrelation, also reduced alcohol intake. The observations mimic the studies by Rao *et al*. who showed that ceftriaxone administered systemically increased GLT1 levels and markedly reduced chronic ethanol intake (Rao *et al*. 2015). It is noted that both systemic ceftriaxone and intranasally administered exosomes activated GLT1 and inhibited ethanol intake within 24 to 48 hours. Unlike the findings of Rao *et al.* (2015), increases in GLT1 were not observed in prefrontal cortex, but only in nucleus accumbens. An unexpected observation, while also reproducing the effect of ceftriaxone (Leclercq *et al*. 2012; Rao *et a*l. 2015), is the increase induced by exosomes on the transporter xCT, which delivers glutamate into the extracellular space. However, the relative glutamate transport stoichiometry of these two carriers is not known. Further studies are necessary to unravel the physiological/pharmacological meaning of this

observation. It is also noted that the exosome effects on xCT were observed in nucleus accumbens, but not in prefrontal cortex unlike that observed for the ceftriaxone studies by Rao *et al*.(2015). As described by Trotti *et al*. (1998) the determination of GLT-1 by Western Blot is subject to experimental error depending on whether the protein sulfhydryl groups are or not fully reduced.   Changes in tissue oxidative stress are expected to alter the Western banding.   Further 4-hydroxynonenal (4-HNE), known to be increased in oxidative stress conditions, forms adducts with GLT1 (Lauderbck *et al*. 2001).   The administration of exosomes led to clear increases in GLT1 expression (mRNA) which are not subject to these experimental problems

For the GSSG/GSH studies we focused on the hippocampus as it is one of the areas most affected in human alcoholism. Also since hippocampus receives drug cued-input, which maintains drug intake, and projects into the nucleus accumbens (see Scofield *et al.* 2016). In the case of GLT1 expression we focused on nucleus accumbens and prefrontal cortex since it has been previously reported that chronic alcohol consumption alters GLT1 levels in these brain areas (Sari *et al*. 2013)

As indicated, exosomes administered via the intranasal route and labelled with a fluorescent dye were found in the brain within the first 24 hours; in line with the marked inhibition of ethanol intake. Exosomes were found in the cell cytoplasm, in different brain areas. Exosomes were also observed in the brain vascular space, such that a paracrine (or systemic) effect may not be ruled out.

Overall, studies presented suggest that the intranasal administration of exosomes derived from activated human mesenchymal stem cells markedly inhibits chronic alcohol intake and post-deprivation alcohol relapse. Increases in glutamate transporter expression, counteracting the inhibition of glutamate transporter ability due to increased oxidative stress conditions may constitute the likely mechanism of inhibition of ethanol intake by exosomes.

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AUTHOR CONTRIBUTIONS

FE, MEQ, PM, YI contributed to research design. FE, MEQ, PM, DS, ME, MK, ES-H MH-M contributed to data generation. FE, YI drafted the manuscript. All authors reviewed and approved the manuscript.

LEGENDS TO FIGURES

Figure 1.

Characterization of exosomes from activated human adipose tissue-derived mesenchymal stem cells (AD-MSCs).

(A) NanoTracking System Analysis showing size distribution of microvesicles isolated from activated human AD-MSCs ranging between 70 to 200 nm, with the major concentration in the range of 100 to 120 nm. (B) Transmission electron microscopy image showing integrity of the exosome sample (scale bar 100 nm). (C) Western blot analysis showing enrichment of the classic exosome markers GM130, ALIX, Flotilin and EpCAM, in the exosome fraction compared to the whole MSC lysate. Absence of calnexin and HSP60 markers in the exosome fraction was used to discard endoplasmic reticulum contamination. Results are representative of three independent experiments.

Figure 2.

Intra-cerebroventricular administration of MSC-derived exosomes inhibits chronic ethanol intake.

Voluntary ethanol intake of animals that had consumed ethanol for 16 weeks and were intra-cerebroventricularly injected with a single dose of exosomes from activated human AD-MSCs (1.5×10^9) or vehicle. Ethanol intake is expressed as g of ethanol consumed/kg body weight/day. Data are presented as mean ± SEM of 6 animals per experimental condition (* = p < 0.0001; repeated measures ANOVA).

Figure 3.

Intranasal administration of MSC-derived exosomes inhibits chronic ethanol intake and relapse-like ethanol intake in the ethanol post-deprivation condition (ADE).

(A) Voluntary ethanol intake of animals that had consumed ethanol for 13 weeks and received three intranasal doses of exosomes derived from activated human AD-MSCs (1.5×10^9 exosomes each dose) or vehicle. Ethanol intake is expressed as g of ethanol consumed/kg body weight/day. (B) Voluntary water intake of the same animals of figure A. Data are expressed as mL of water consumed/kg body weight/day. (C) Seventeen days after the administration of the first intranasal dose of exosomes or vehicle a 14-day withdrawal period was imposed. During the withdrawal period animal received two intranasal doses of exosomes following the weekly administration schedule. Relapse drinking after the deprivation period was determined by allowing animals a 60-minute ethanol re-access. Ethanol intake during the 60-minute re-access is expressed as g of ethanol consumed/kg body weight/60 minutes. (D) Blood ethanol level evaluated after the 60-minute relapse-like ethanol consumption. Data are presented as mean ± SEM of 6 animals per experimental condition (* = $p < 0.01$; repeated measures ANOVA for figures A and B and $p < 0.05$, student t test for figures C and D).

Figure 4.

 Intranasal administration of MSC-derived exosomes normalizes ethanol –induced astrocyte (GFAP) activation and microglial density (Iba-1).

Intranasal administration of MSC-derived exosomes astrocyte (GFAP) and microglial (Iba-1) density was evaluated immediately after the 60-minute relapse-like ethanol consumption. (A) Representative microphotographs of GFAP (red, upper panel) and Iba-1 (green, depicted by arrows, bottom panel) immunoreactivity counterstained with DAPI (blue, nuclear marker) evaluated in hippocampus of animals intranasally administered five doses of MSC-derived exosomes or vehicle. Animals consuming only water were used as control. Scale bar 25 μm. (B) Quantification of the length of astrocytic process. (C) Quantification of the thickness of primary astrocytic process. (D) Quantification of microglial density. Data were evaluated by confocal microscopy and FIJI image analysis software. Data are shown as mean ± SEM. N = 5 per experimental condition. All determinations; *$p < 0.05$. ANOVA and Bonferroni post-hoc test.

Figure 5.

 Intranasal administration of MSC-derived exosomes reduces ethanol-induced oxidative stress (GSSG/GSH) in hippocampus of rats following a 60-minute ethanol re-access.
Oxidative stress (GSSG/GSH) in hippocampus was evaluated immediately after the 60-minute relapse-like ethanol consumption. GSSG/GSH ratio in hippocampus samples of animals intranasally administered five doses of MSC-derived exosomes or vehicle. Animals

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consuming only water were used as control. Data are presented as mean ± SEM of 6 animals

per experimental condition (* = p<0.01, one way ANOVA).

Figure 6.

Rapid inhibition of ethanol intake and hippocampal oxidative stress (GSSG/GSH ratio)

induced by single intranasal administration of MSC-derived exosomes. (A) Voluntary

ethanol intake of animals that had consumed ethanol for 13 weeks and received a single

intranasal dose of exosomes derived from activated human AD-MSCs (1.5×10^9 exosomes) or

vehicle. Ethanol intake is expressed as g of ethanol consumed/kg body weight/day. (B)

GSSG/GSH ratio in hippocampus samples evaluated two days after the intranasal

administration of a single dose of exosomes or vehicle. Animals consuming only water were

used as control. Data are presented as mean ± SEM of 6 animals per experimental condition

(* = p < 0.01; repeated measures ANOVA for figure A and p<0.01, one way ANOVA for figure

B).

Figure 7.

Intranasal administration of MSC-derived exosomes increases glutamate transporters

expression in nucleus accumbens. The mRNA levels of glutamate transporters GLT1, GLT1a,

GLT1b, xCT and GLAST were determined by quantitative RT-PCR in nucleus accumbens and

prefrontal cortex of chronically ethanol consuming rats two days after the intranasal

administration of a single dose of exosomes derived from activated human AD-MSCs

(1.5×10^9 exosomes) or vehicle. Data were normalized against the mRNA levels of the

housekeeping genes β-actin and GAPDH. Animals consuming only water were used as control. Data are presented as mean ± SEM of 6 animals per experimental condition (* = p < 0.05; one way ANOVA).

Figure 8.

Intranasal administration of human AD-MSCs derived exosomes is an efficient route to reach the brain. (A) PKH26 fluorescent from labeled exosomes observed in dorsal, ventral and sagittal views of the brain of animals that consumed ethanol for 13 weeks, 2, 6, 12 and 24 hours after a single intranasal dose of PKH26-labeled human derived AD-MSCs exosomes (1.5×10^9). Saline or PKH26-saline administrations were used as control. PKH26 fluorescence was evaluated with an *In Vivo* MS FX PRO image system. A fluorescence chart is shown as a side bar. (B) Representative microphotographs showing fluorescence of labeled exosomes in the perinuclear cytoplasm of a cell, and in the extracellular space. Sagittal sections (30 μm) of the brain were stained with DAPI (4',6-Diamidino-2-Phenylindole, blue), as a nuclei marker and examined by confocal microscopy (Olympus FV10i). Microphotographs were taken from regions showing high red PKH26 fluorescence when examined with the In Vivo FX PRO image system (Fig. 8B). Representative microphotographs taken from ventral striatum are shown, 12 h after intranasal saline +PKH26 (a-c) or MSC derived exosomes +PKH26 administration (d-f). PKH26 positive exosomes (red fluorescence) can be seen in the cell cytoplasm of a neuron (white arrow), with a reticular distribution, suggesting cellular exosomes uptake. Fluorescence was also observed in glia (not shown). PKH26 positive exosomes were [can] also [be] seen in the extracellular space. Similar results were observed in sections from

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olfactory bulb, cerebral cortex, dorsal striatum, thalamus, hypothalamus and brainstem. Bar

Scale=25μm

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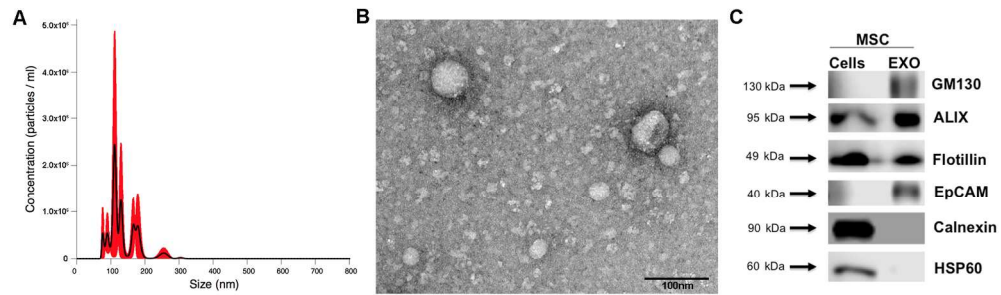
Figure 1

176x51mm (300 x 300 DPI)
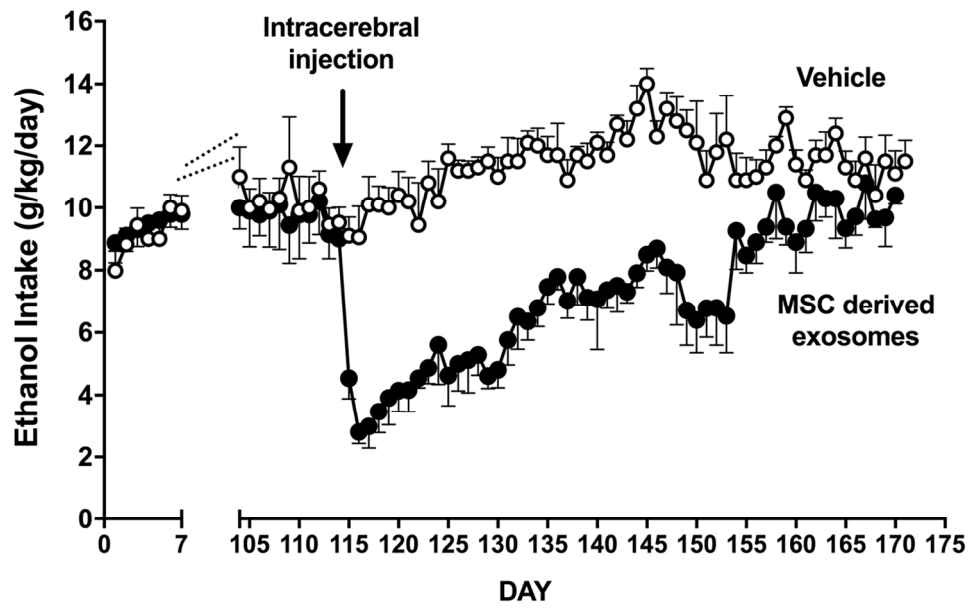
Figure 2

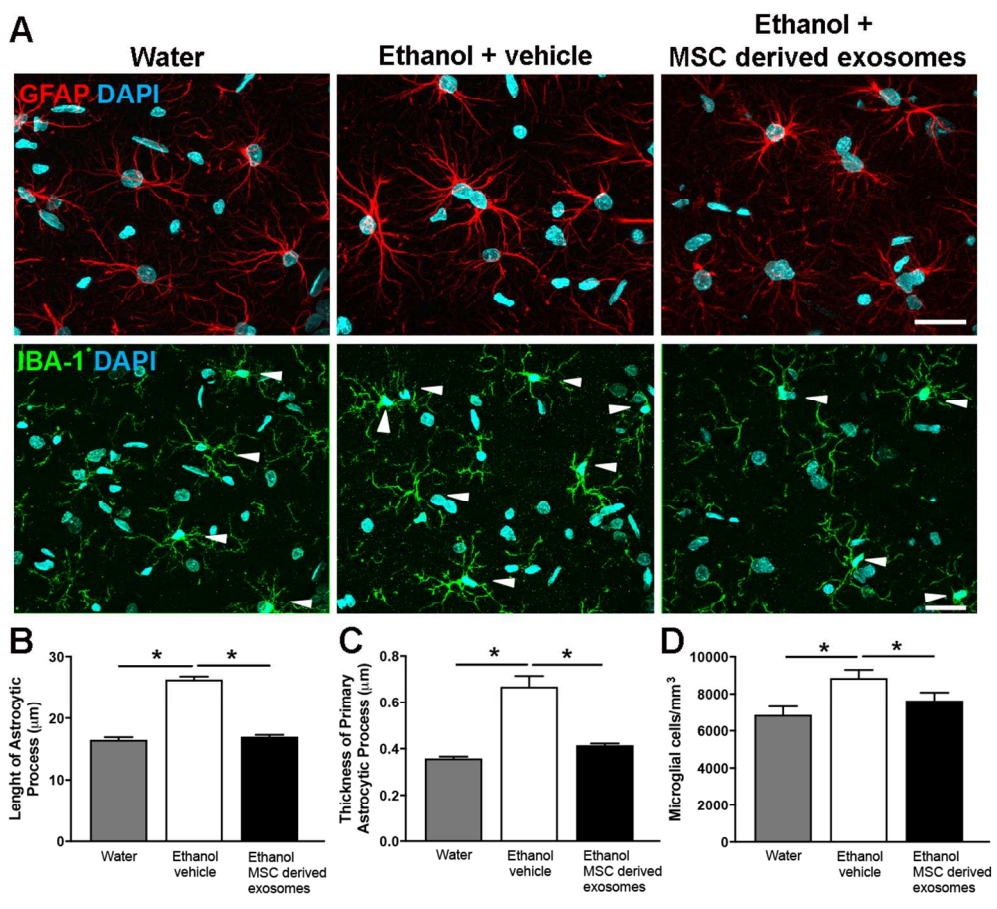120x75mm (300 x 300 DPI)
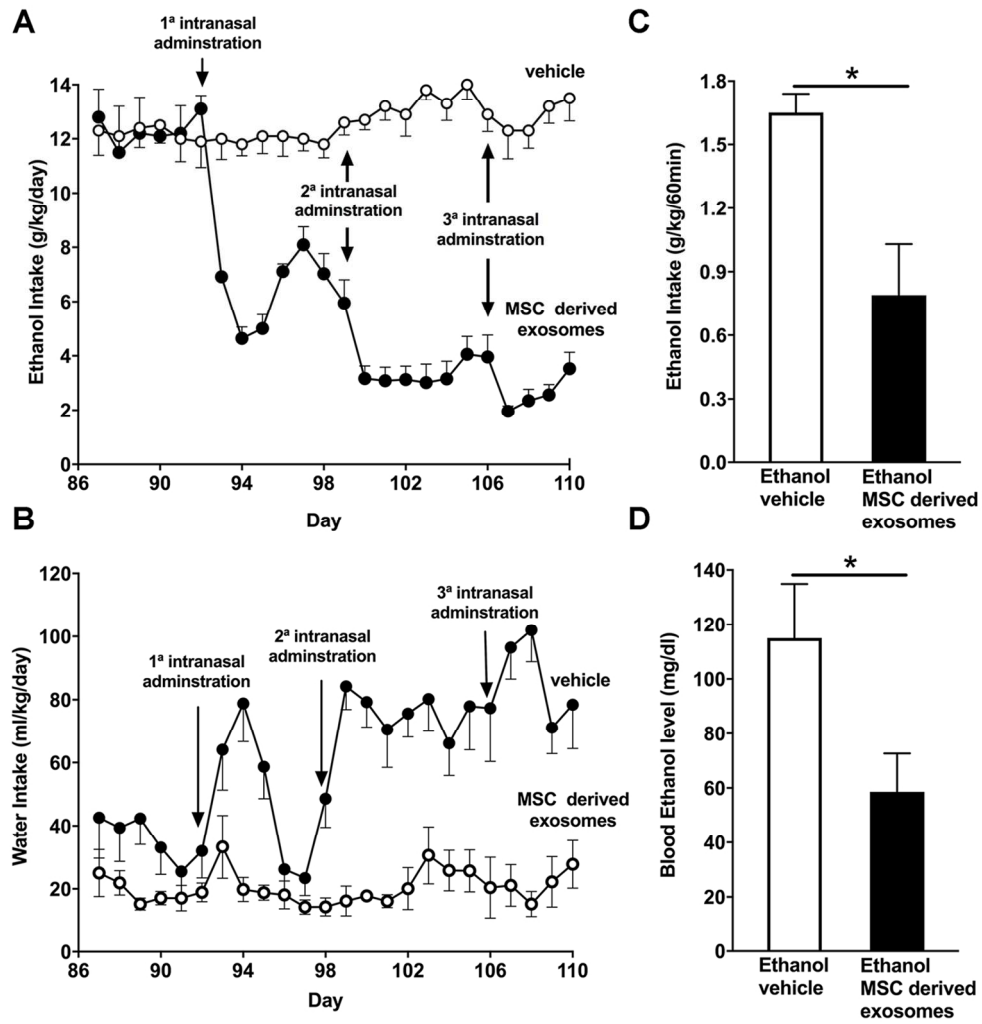
Figure 3

119x125mm (300 x 300 DPI)

Figure 4

109x99mm (300 x 300 DPI)
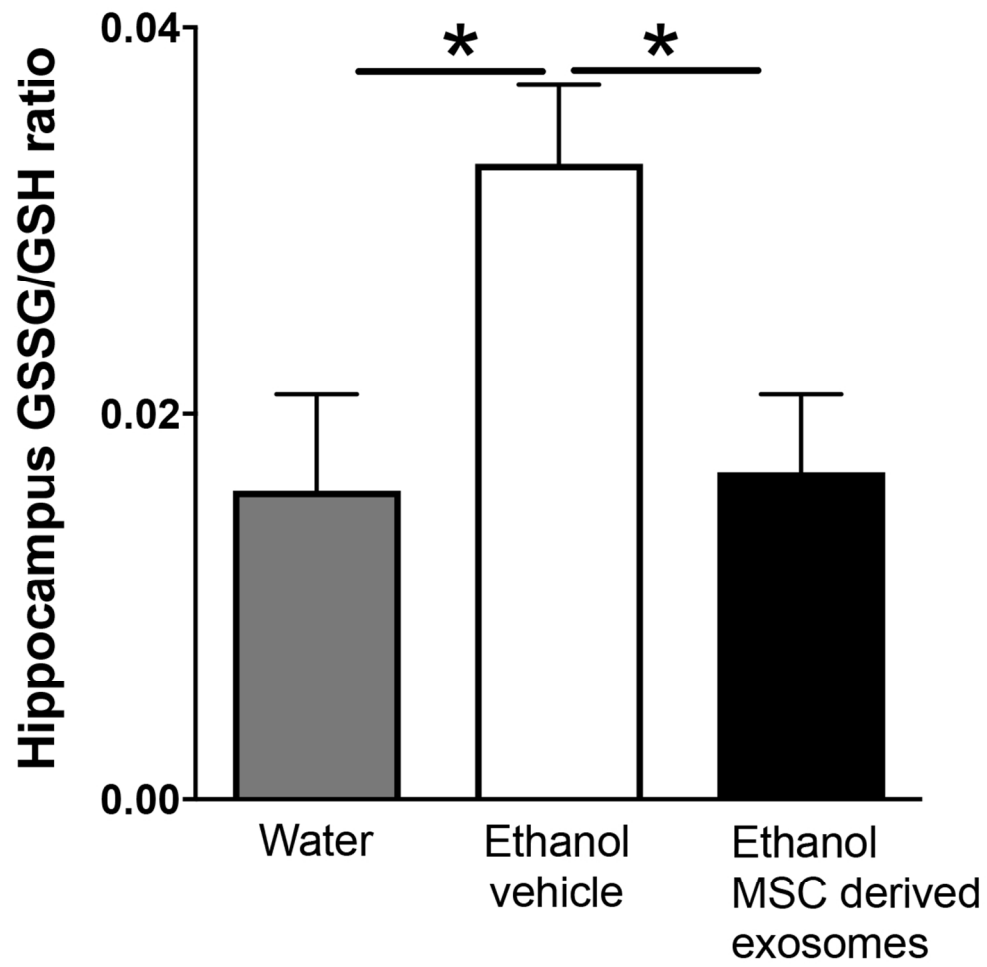
Figure 5

99x99mm (300 x 300 DPI)
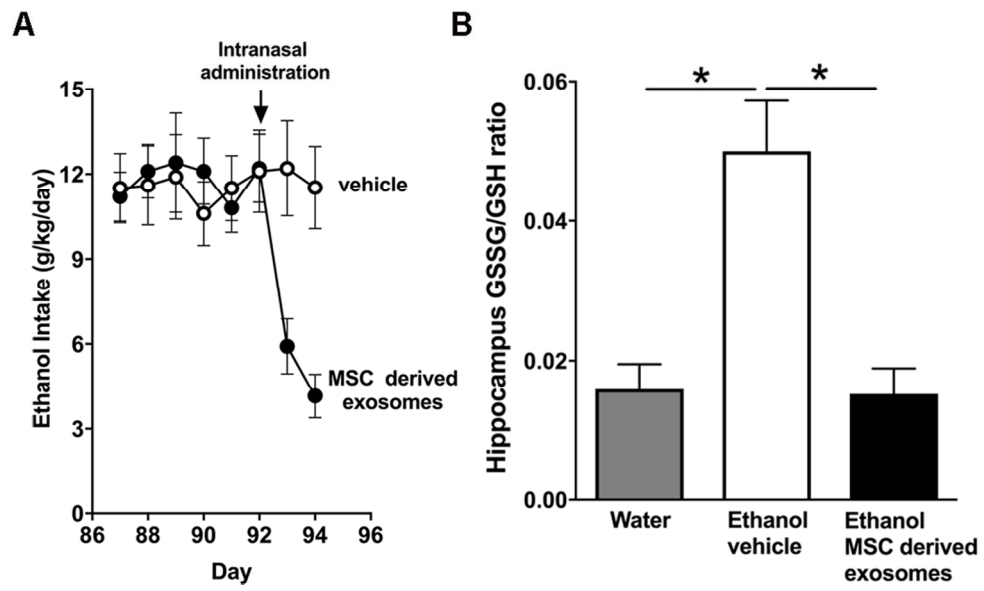
Figure 6

89x53mm (300 x 300 DPI)
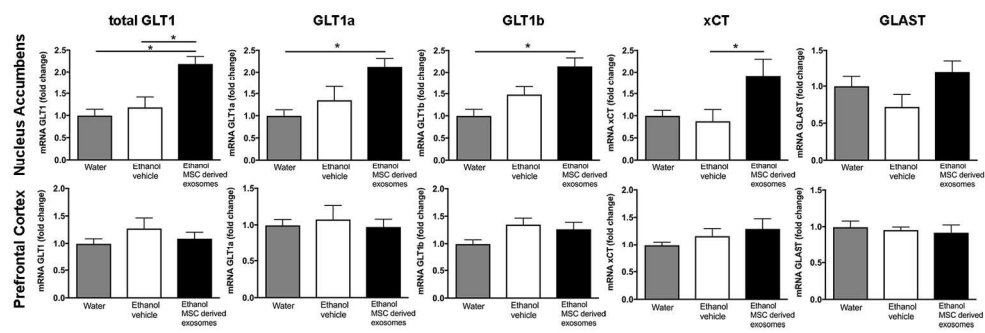
Figure 7

159x53mm (300 x 300 DPI)

A

Saline | Saline + PKH26 | MSC derived exosomes + PKH26 (2h, 6h, 12h, 24h)

Dorsal view

Ventral view

Sagittal view

Fluorescence intensity: 1,300 — 1,100 — 984 — 825
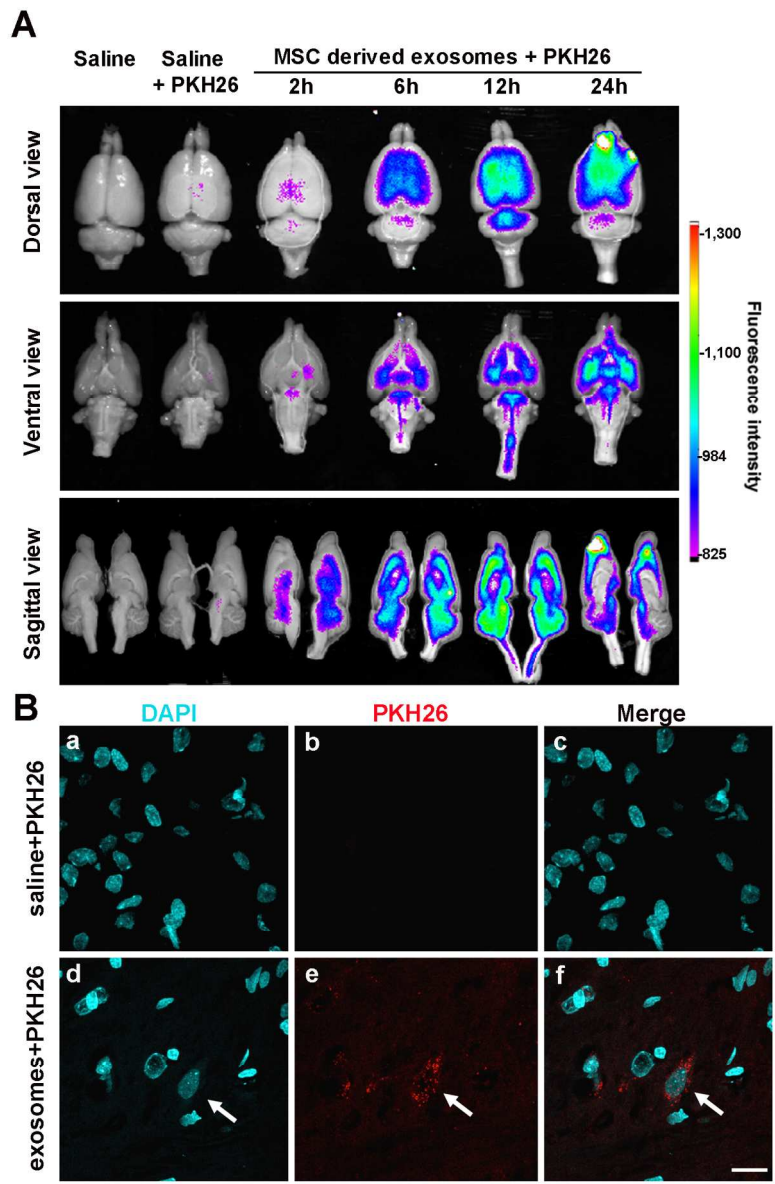
B

DAPI | PKH26 | Merge

saline+PKH26: a, b, c

exosomes+PKH26: d, e, f

Figure 8

129x197mm (300 x 300 DPI)