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No evidence for exosome treatment in reducing alcohol relapse – a failed confirmatory multi-center study

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ABSTRACT

Preclinical studies have suggested that intranasal administration of mesenchymal stem cell-derived exosomes can reduce alcohol intake and relapse-like behavior in rodents. To assess the translational potential of these findings, we conducted a preregistered, multi-center preclinical randomized controlled trial (preRCT) across several German research sites. Using the alcohol deprivation effect (ADE) model in both male and female Wistar rats, we evaluated whether exosome treatment attenuates relapse-like drinking behavior following repeated deprivation phases. Given the hurdles in standardizing exosome isolation, dosage, and intranasal delivery into the brain, we performed first a functional validation experiment of intranasally delivered exosomes. For providing such a functional proof, we conducted electrocorticography (ECoG) recording and showed that exosomes are capable of mitigating impaired electrophysiological activity in the prefrontal cortex in rats that underwent the ADE procedure. However, contrary to previously published positive findings, our confirmatory multi-site preRCT results did not demonstrate a significant reduction in ethanol consumption during an ADE in Wistar rats following exosome treatment. No sex or site-specific effects were observed. Given the absence of efficacy, the study was terminated early in alignment with the 3R principles of animal research ethics. These findings suggest that the previously reported benefits of exosome treatment may be model-specific and do not generalize to a broader genetic background or experimental model. Our results emphasize the necessity of replication across diverse preclinical models and rat lines prior to clinical translation and highlight the importance of publishing null results to improve transparency and reproducibility in addiction research.

The field of psychiatry continues to face major challenges in translating preclinical findings into effective clinical treatments. Despite decades of promising results in animal models, many pharmacological candidates fail during clinical trials, leading to a stagnation in therapeutic innovation and diminishing interest from the pharmaceutical industry. Contributing to these translational failures are several methodological limitations in preclinical research, including the predominant use of male animals, lack of chronic administration studies, insufficient attention to reproducibility, and poor alignment with human disease phenotypes [1]. Recent developments in the field underscore the urgent need for robust, generalizable, and clinically relevant preclinical study designs [2,3]. In response, initiatives such as the

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STRINGENCY framework have emerged, advocating for higher methodological standards, including the use of multi-center preclinical randomized controlled trials (preRCTs) to enhance reliability and translational potential of findings [4]. Recently, we published the pre-RCT approach as an integral module in the drug development process for novel psychiatric medications. We demonstrated the utility of this pre-RCT method by testing enantiomers of ketamine in an animal model of alcohol relapse [5]. With this proof-of-principle achieved through a multi-center pre-RCT, we are now well-positioned to effectively evaluate the efficacy of new neuromodulatory or pharmacological interventions for Alcohol Use Disorder (AUD).

One promising innovative intervention involves mesenchymal stem cell (MSC)-based therapy. MSCs are adult stem cells recognized for their remarkable capacity for self-renewal and multipotent differentiation. The administration of MSC-derived components, specifically exosomes, is believed to exert significant anti-inflammatory and antioxidant effects, providing a multi-targeted approach to mitigate the neuropathological consequences of chronic alcohol consumption [6].

Recent research by Ezquer et al. [7] demonstrated that intranasal delivery of MSC-derived exosomes significantly reduced ethanol consumption and dramatically suppressed relapse drinking in UChB rats [8]. These findings highlight a novel therapeutic strategy for AUD, primarily by addressing oxidative stress and neuroinflammation. However, given the broader context of translational bottlenecks in psychiatric research, independent replication in diverse and clinically relevant models is essential before advancing to clinical application [4,9].

To address this need, we conducted a multicenter confirmatory preRCT and designed to replicate and expand upon the findings of Ezquer et al. using the alcohol deprivation effect (ADE) model in both female and male Wistar rats. Following repeated deprivation phases, the ADE is characterized by an increased demand for alcohol intake that results in compulsive drinking behavior [10–14]. The model resembles a relapse situation in patients with AUD and has been validated by the effectiveness of approved anti-relapse compounds such as acamprosate and naltrexone [15–17].

The study was designed to be executed at three independent German research sites—Mannheim, Dresden, and Erlangen—and adhered to stringent quality criteria as detailed in our preregistration (DOI: 10.17590/asr.0000111). We applied the same exosome isolation protocol, the same dosage, and the same intranasal exosome administration protocol as Ezquer et al. [7] to confirm that intranasal delivered exosomes reduce relapse-like drinking behavior in Wistar rats following alcohol deprivation. Given the hurdles in standardizing exosome isolation, dosage, and intranasal delivery into the brain, we recognized the necessity of providing a functional validation of intranasally delivered exosomes' efficacy. For providing such a functional proof we implanted a neuroprosthetic interface, previously established at the Dresden site [18,19], to perform electrocorticography (ECoG) recording directly from the surface of the medial prefrontal cortex - an area critically implicated in craving and loss of behavioral control in substance addiction e.g. [20,21]. Our recent studies demonstrated that compounds with potential anti-relapse properties, such as metabotropic glutamate receptor 2 (mGlu2) agonists, can restore impaired ERO activity in the prefrontal cortex of rats subjected to our alcohol deprivation effect (ADE) protocol. Furthermore, mGluR2 agonists have been shown to effectively reduce relapse-like drinking behavior in the ADE model [22]. Based on these findings, we hypothesized that if exosomes are capable of mitigating impaired electrophysiological activity in the prefrontal cortex within the ADE model, they could be deemed functionally active and potentially effective in reducing ADE.

Methods

Animals and ADE procedure

Two-month-old male and female Wistar rats were used including a total of 53 female and 60 male and exactly the same experimental conditions were applied at all research sites (Mannheim; n=80 rats, Dresden; n=33 rats, Erlangen; n=0 rats).

Housing and husbandry CIMH Mannheim

For the study, we used original Makrolon cages type III, dimensions mm: top (outside) 425 × 276, bottom (inside) 390 × 230, height: 153mm, floor area: about 820cm2 with feeders at the cover and followed by a 5cm raised area. Rats for the alcohol drinking experiment were single housing for the duration of the experiment. Bottles hold 250ml with ball nipples. Feeding practice was ad libitum (LASQCdiet® Rod16-Auto, Lasvendi) and soy-free. The dark/light cycle was 12:12 with lights on 6am and off at 6pm, the light intensity was max 130lux in the drinking room and 50 lux within every cage. The relative humidity in all rooms was 45-65% and room temperature was constant between 22-24°C. Bedding material in the cages had steam-sterilized aspen wood (2-3mm, Abedd) with no environmental enrichment. Cages were changed once per week.

Housing and husbandry medical faculty, TU Dresden

For the study, we used original Makrolon cages type III, dimensions mm: top (outside) 425 × 276, bottom (inside) 390 × 230, height: 153mm, floor area: about 820cm2 with feeders at the cover and followed by a 5cm raised area. Rats for the alcohol drinking experiment were single housed for the duration of the experiment. Bottles hold 300 ml with ball nipples. Pelleted food (V1534-300, ssniff Spezialdiäten GmbH, Soest, Germany) and water were available ad libitum. The dark/light cycle was 12:12 with lights on 6 am and off at 6 pm. The relative humidity in all rooms was about 40 – 55 % and room temperature was on average 20 - 22°C. Rats were housed on sterilized sawdust bedding (Ssniff - Bedding 3/4 S, Altrogge, Lage, Germany) and with Bed-r'Nest material (Datesand Ltd., Bredbury, UK). Cages were changed once per week.

All experiments were approved by the institutional Committees on Animal Care and Use, by the respective Regierungspräsidium of each site, and were performed in accordance with the European and German national guidelines. To model relapse-like drinking in rats we applied the ADE model as previously published [10,13]. After two weeks of habituation to the animal room, rats were given *ad libitum* access to tap water and to 5%, 10% and 20% ethanol solutions (v/v) as well. Spillage and evaporation were minimized by the use of special bottle caps. With this procedure the ethanol concentration remains constant for at least one week [23]. The positions of bottles were changed weekly. The first deprivation period was introduced after eight weeks of continuous alcohol availability. After a deprivation period (between 3-4 weeks), rats were given access to alcohol again and two more deprivation periods were introduced in a random manner, i.e., the duration of following drinking and deprivation phases was irregular, i.e. approximately 4±1 week and 2±1 week, respectively in order to prevent adaptive behavioral mechanisms [24,25]. The long-term voluntary alcohol drinking procedure including all deprivation phases lasted a total of 10 months.

Random allocation of rats to the treatment arms was done separately for each strata (formed by sex and participating center) using computer-generated lists of permuted blocks (randomization schedule was generated using a custom developed R script resulting in such a way that the mean baseline total alcohol intake was approximately the same in each group). Experimenters had been blinded for the treatment using the EQIPD blinding protocol[1] and the blinded code was only broken after the analysis of the data was completed.

From the 60 male rats, 10 rats that received exosome treatment as well as 10 rats that received vehicle treatment were operated following

[1] https://paasp.sharepoint.com/:w:/s/EQIPD/EZbRvZmZGoRGtsjc1Wk_XOsBLsnNAbg-FBFVj9h199oYMA?rtime=0Pyue-3N3Eg

the ADE procedure and used for electrocorticography (ECoG) recordings of neural activity (n=9/group had functional electrodes for recodings).

ECoG recording and acute exosome modulation of neural activity

Micro-EcoG interfaces, so-called neuroprosthetic interfaces, were manufactured as previously described [18,19,26]. During the final two-week period of alcohol deprivation (6th deprivation), the animals were habituated to the recording set-up and underwent surgery to implant the neuroprosthetic device. Stereotactic implantation was performed under subcutaneous (s.c.) anaesthesia (fentanyl (0.005 mg/kg, Hameln Pharma), midazolam (2 mg/kg, Ratiopharm), and medetomidine hydrochloride (0.135 mg/kg, Orion Pharma) as previously described in detail [18,19,26].

ECoG recordings were implemented three days after the animals had undergone surgery. One animal at a time was placed in a rodent sling (Lomir Biomedical Inc.) to reduce movement artefacts within an electrically shielded and sound-insulated audiometry booth. Recordings were performed at a sampling rate of 3 kHz using the Intan RHD2000 USB interface system cable connected to the implant plug-in module. In order to elicit event-related oscillatory (ERO) activity an auditory oddball paradigm was used as previously described [19].

Data processing was performed using the EEGLAB toolbox [27] (Version 2019.1) for Matlab. Following offline filtering using a 0.1 – 45 Hz bandpass finite impulse response filter (Kaiser windowed, Kaiser β = 5.65, filter length 54330 points) and interpolation of dysfunctional channels data were segmented in epochs between -100 and 700 ms relative to stimulus onset separately for standard and deviant sounds and baseline-corrected using the pre-stimulus interval between -100 ms to 0 ms. Noisy epochs and artefacts were identified and excluded based on a delta criterion of 500 μV and visual confirmation before averaging epochs for single subjects and over all animals (grand average). As neural responses to the frequent standard sounds rarely display pronounced neural responses, indicating habituation effects due to the high repetition rate [19], all analyses were performed on the deviant-minus-standard difference responses.

Oscillatory power in the delta (1 – 4 Hz), theta (4 – 8 Hz), alpha (8 – 12 Hz), beta (12 – 30 Hz) and gamma (30 – 45 Hz) bands was determined by applying the function pop_newtimef.m in EEGLAB based on a Fast Fourier transform using 400 data points and a pad ratio of 64. The resulting event-related spectral perturbation (ERSP) was calculated as decibels (dB) ($\triangleq$ 10*log10 (μV2/Hz)). Recordings were conducted exclusively in male animals (n = 10 per group) as the dimensions of the neuroprostheses have thus far been optimized for male rat brain anatomy only.

Design of the multi-site preRCT

For the present study we implemented the novel concept of randomized multi-center preclinical phase II testing in laboratory animals [5]. Trial preregistraition was done ahead of the study (DOI: 10.17590/asr.0000111). We structured the materials and methods section following the SPIRIT recommendations (https://spirit-statement.org/) that are used for clinical studies.

Inclusion/ exclusion criteria

The key inclusion criterion was a daily baseline alcohol intake of at least 2.5g/kg/day to ensure relevant alcohol intake of rats. Exclusion criteria included adverse effects in animal behavior (with the exception of the alcohol-related phenotype) or any other pathological features (e.g. tumors). The GV-SOLAS recommended score sheet[2] was used to determine exclusion of rats during the study. Other exclusion criteria involved leaky bottles or incorrect study drug application.

Groups

As preclinical and clinical evidence suggests that sex influences disease trajectories and interventions in AUD patients [28], male and female rats were studied in comparison in our two-arm design: placebo vs. the application of exosomes of 160 μl (containing 7.5 μg of exosomes derived from activated human adipose tissue-derived MSCs). As placebo control we used, PBS (Phosphate buffered saline, pH 7.4), as exosomes were diluted in PBS solution. We followed exactly the published protocol by Ezquer et al. [7] for the isolation of MSC-derived exosomes (see suppl. Information).

Interventions

In order to study the effects of exosomes in comparison to vehicle, rats were divided into groups in such way that the mean baseline total alcohol intake was approximately the same in each group. Baseline drinking was measured daily for one week. After the last day of baseline measurement, the alcohol bottles were removed from the cages, leaving the rats with free access to food and water for the complete deprivation period.

During the 5th period of abstinence drug testing was performed before the 6th ADE measurement (Fig. 2A). Repeated deprivations have been shown to boost compulsive relapse behavior [10]. Two intranasal doses of exosomes of 160 μl (containing 7.5 μg of exosomes derived from activated human adipose tissue-derived MSCs in PBS divided into two aliquots; one for each nostril) were given at weekly intervals within the 5th period of abstinence. We conducted preliminary experiments to refine the final application regime. These experiments showed that the intranasal administration procedure under anesthesia 24 hours opposed to 72 hours before relapse (see supplementary Fig. 8), produced unspecific behavioral inhibition that itself suppressed alcohol drinking. By suspending treatment 3 days before reintroducing the bottles, we aimed to separate such unspecific acute effects from the specific, longer-lasting impact of exosomes on relapse-like drinking. Therefore, re-access to ethanol was allowed 3 days after the last intranasal administration of exosomes. The application of exosomes was done between ZT03 to ZT06 (the terminology of time points is according to a standardized notation, the so-called “zeitgeber time” where ZT0 is the beginning of the light phase and ZT12 the beginning of the dark phase) [29]. For the application each rat was separately transferred from the alcohol drinking room to the intervention room. Rats were anesthetized with 1 mg/kg of domitol (i.p.) and placed in a supine position. The experimenter applied the exosomes bilaterally on the rhinarium, the area referred to as the glaberous skin around the nostrils, using a 20μl pipette. Twenty microliters of exosome solution were distributed with the tip of the pipette and allowed to diffuse into the squamous epithel of both the left and right rhinarium every 5 minutes (four times in each nostril) into alternative sides 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 1.0×10^6 MSCs) was delivered. At the end of the procedure all rats were administered with 5 mg/kg (s.c.) of antisedan to counteract the effect of dormitol. The alcohol bottles were reintroduced 72h after the second exosome administration and the occurrence of an ADE was determined. Total ethanol (g/kg of body weight/day) and water intake (ml/kg of body weight/day) were measured daily for the subsequent week.

Outcomes

Primary. The primary endpoint (efficacy of the treatment with reduction of relapse) was defined as the reduction of ADE in the first 5 days after re-access to alcohol after treatment.

Secondary outcomes. Secondary endpoints were sex differences in the

[2] https://www.gv-solas.de/wp-content/uploads/2020/02/Moeglichkeiten-der-Belastungsbeurteilung-im-Tierversuch_2020.pdf

efficacy of exosome treatment.

Sample size

The experimental unit of this study was the individual rat. Accordingly, data taken from different animals are considered throughout as values of mutually independent random variables, and correlations are assumed to exist only between observations taken at different points in time from the same animal. In the study by Ezquer et al., 2018 (6 animals per experimental group), the effect size defined as the standardized difference of mean ADE under drug intervention was estimated to be $d = 1.41$. Sample size planning was then done against the alternative that the true effect size coincides with the lower 90%-confidence bound associated with that value, which was computed by means of exact methods to be 0.54391. To ensure that an effect size of that magnitude can be detected in a two-sample t-test at 1-sided significance level $\alpha = 0.05$ with power $1\text{-}\beta = 0.90$, a sample size of $n = 59$ per group is sufficient (as can be shown making use of the exact algorithm for sample-size calculation based on the non-central t-distribution). To ensure that each of the strata defined in terms of center and sex shall be equally represented in both arms of the trial an n of 20 per site (per arm) was proposed.

Compounds

Alcohol drinking solutions were prepared from 96% ethanol and then diluted with tap water. Exosomes production was done as published in Ezquer et al., 2018 and described in detail within the supplementary material section (see suppl. Fig. 1-3). Final dilution was with PBS (Sigma-Aldrich, Germany). In order to confirm the *in vivo* efficacy of the here used exosome batch a validation experiment with female UChB rats selectively bred for alcohol preference [30], were used.

Statistics

Data derived from home-cage drinking (total alcohol intake) were analyzed using a two-way repeated measures Analysis of Variance (ANOVA), with treatment as the between-subject factor and day/week as the within-subject factor. Data from males and females were pooled and sex as well as site was added as covariate. We did not have any missing data points of any animal that went into the analysis. In general, we tested data for skewness using the Shapiro-Wilk test and repeated measures ANOVA were tested for sphericity using Mauchly's test. When appropriate, corrections using Greenhouse-Geisser for $\varepsilon < 0.75$ and Huynh-Feldt for $\varepsilon > 0.75$ were performed. To visualize differences between sexes, individual subgroup graphs for each sex are provided in each experiment. All behavioural statistical analyses were conducted with Statistica 13.3 (Statsoft, Hamburg, Germany). Statistical analyses for neural activity patterns were conducted using R (Version 4.2.3, R Foundation for Statistical Computing). Differences in ERO activity (max. bandpower (ERSP), latency and frequency of max. power within each frequency band) following exosome treatment was examined by applying a one-way analysis of variance (ANOVA) with between-factor group (vehicle vs. exosome treatment).

Results

To ensure the authentication of biological resources used for the study, the generation and characterization of the study drug (exosomes) is described within the supplemental material section (supplementary Fig.s 1-4). Especially, supplementary Fig. 4 demonstrates that the produced exosomes for the current study are effective *in vivo* in reducing alcohol intake in UChB rats, thereby also serving as confirmatory data to Ezquer et al., 2018 on the efficacy of exosomes in UChB rats. Additionally, to prove that intranasal delivered exosomes were able to reach the brain, we conducted neural recordings on a subset of 18 male Wistar rats. In rats that derived from the ADE model the gammapower was markedly reduced ($F1,160 = 13.855$, $p < 0.001$, $\eta2 = 0.08$) and peaked significantly earlier ($F1,160 = 9.133$, $p = 0.003$, $\eta2 = 0.054$) than in vehicle-treated animals. Additionally, exosome-treatment strengthened higher theta ($F1,160 = 10.654$, $p = 0.001$, $\eta2 = 0.062$) and gamma ($F1,160 = 33.848$, $p < 0.001$, $\eta2 = 0.175$) frequencies while a profound shift towards lower frequencies was observed in the beta band ($F1,160 = 42.262$, $p < 0.001$, $\eta2 = 0.209$) (Fig. 1A-D, supplementary Fig. 5-7 and files: descriptives and ANOVA). These results provide functional proof that the intranasally applied exosomes indeed reached the brain and were active on prefrontal electrophysiological impairments in Wistar rats subjected to the long-term ADE procedure.

However, contrary to the findings of Ezquer et al. [7], our confirmatory multi-site preRCT results did not demonstrate a significant reduction in ethanol consumption during an ADE in Wistar rats following exosome treatment (Fig. 2B). We observed no change on the ADE compared to control animals. A two-way repeated measures Analysis of Variance for all animals confirmed the null result for the treatment factor [$F(1,112)=0.043$, $p > 0.836$]. The outcome was also similar between sex and different experimental sites did not have an effect on treatment outcome [$F(1, 110)=0.998$, $p=0.319$] (Fig. 2C-H).

This data was generated as part of an interim analysis conducted after the completion of half of the planned study cohort ($n = 113$). Interim analyses are a common practice in clinical trials to monitor data trends and ensure participant safety. In line with this approach, we performed an interim analysis to align with the 3R principles (Reduce, Refine, Replace) of animal welfare and use. Given the complete absence of any observable effect, we decided to terminate the preRCT based on the null results.

Discussion

In the present study, we conducted a preclinical randomized controlled trial (preRCT) to test the hypothesis that intranasally delivered exosomes reduce relapse-like drinking behavior (ADE) in Wistar rats following the last alcohol deprivation. The study was designed as a fully powered, hypothesis-driven confirmatory study aimed at generating robust evidence to inform future clinical translation. Contrary to our expectations, intranasal exosome treatment did not reduce relapse-like drinking behavior. This conclusion was drawn following an interim analysis conducted after 50% of the planned sample size had completed the protocol, in accordance with established ethical and scientific guidelines and in alignment with the 3R principles (Replace, Reduce, Refine). Conducting an interim analysis enabled an early evaluation of therapeutic efficacy while minimizing unnecessary animal use in the absence of a detectable treatment effect. Given the lack of observable benefit at this stage, we made the decision to terminate the study early to avoid further use of resources on a potentially ineffective intervention. Such early-stopping decisions are increasingly encouraged in preclinical research as part of responsible and efficient study design followed by the publication of these negative results. Importantly, the publication of null results should become standard practice to reduce publication bias and promote transparency and reproducibility, as emphasized in the STRIGENCY framework [4].

The discrepancies between the initial data in UChB rats from the Ezquer et al. study [7] may arise due to differences in genetic predisposition to alcohol intake between UChB and Wistar rats [30]. UChB rats are selectively bred from an outbred Wistar colony specifically for high voluntary alcohol consumption, whereas standard Wistar rats exhibit more heterogeneous drinking behavior. Selective breeding of UChB began in the 1950s at the University of Chile (UCh), resulting in the development of two lines: the high alcohol-drinking UChB line ('B' for Bebedoras or Bibulous) and the low alcohol-drinking UChA line. Both lines were derived from a foundation stock of outbred Wistar rats from the Instituto Bacteriológico de Chile. The use of females only in the study by Ezquer et al. aimed to optimize the experimental design by ensuring higher and more stable alcohol consumption relative to body weight, thereby increasing the sensitivity to detect treatment effects. Importantly, male UChB rats were not deliberately excluded from the original

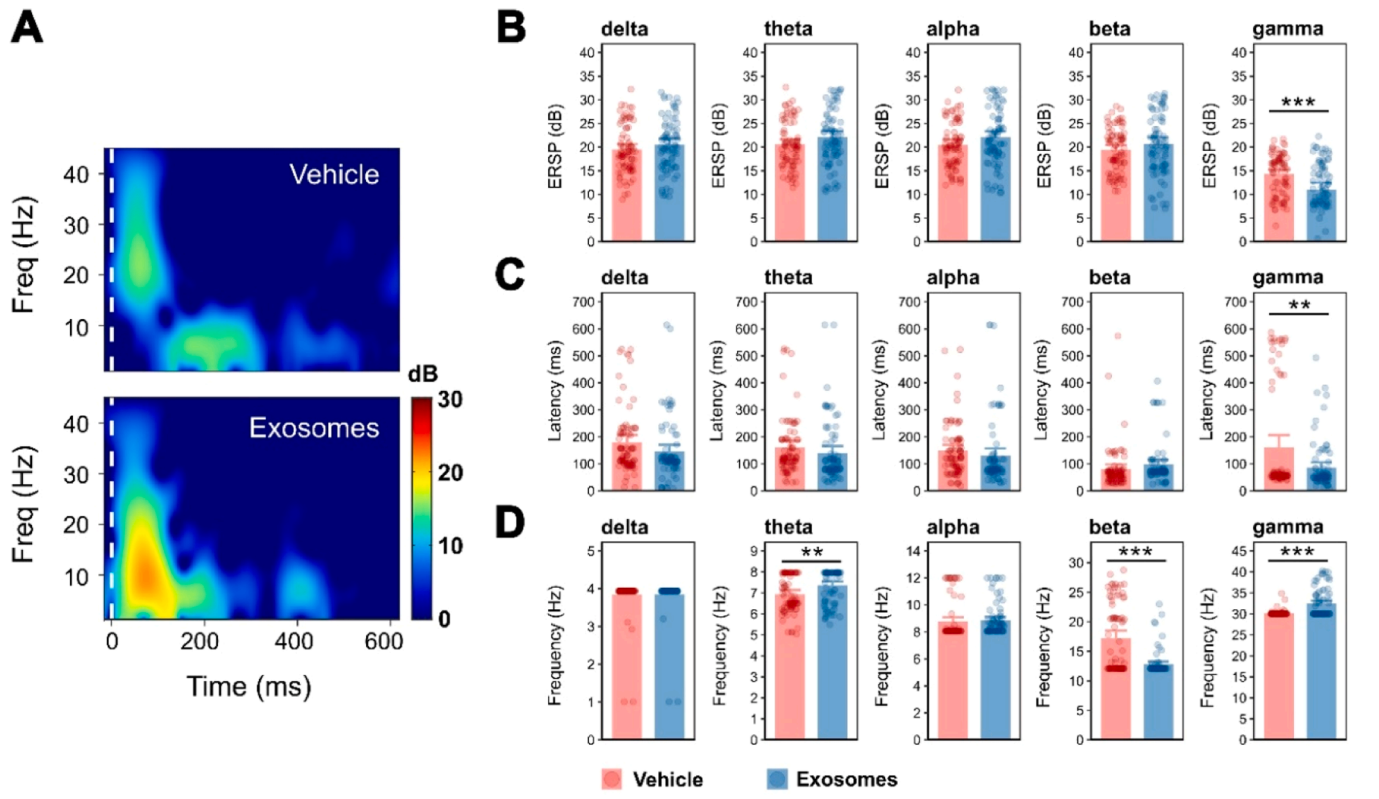

Fig. 1. Impact of single-dose exosome treatment on prefrontal electrophysiological activity in the ADE rat model. A) Grand average deviant-minus-standard auditory event-related oscillatory (ERO) activity, shown for the mediocentral electrode. **B)** Maximum ERO activity, **C)** Latencies and **D)** Frequencies of maximum ERO activity within delta (1-4 Hz), theta (4-8 Hz), alpha (8-12 Hz), beta (12-30 Hz) and gamma (> 30 Hz) frequency bands. Data for vehicle-treated (red) and exosome-treated (blue) animals are displayed as data points for all channels of all rats and mean barplots ± 95 % confidence interval. Asterisks indicate significant differences between groups with $^{**}p < 0.01$, $^{***}p < 0.001$.

study design, and it is likely that they would have responded similarly to the intervention.

One important factor is that UChB rats possess a very specific gut microbiome that has been shown to contribute to their high drinking phenotype [31,32]. Of note, a recent study showed that fragments (microvesicles) of microbiota from UChB rats administered to naïve Wistar rats increase by 5-fold their voluntary intake [33]. This distinct microbiome composition may play a crucial role in modulating the effects of exosome treatment, possibly by influencing neuroimmune interactions and metabolic pathways associated with alcohol consumption. Given that UChB rats show pronounced neuroinflammation and gut dysbiosis—both of which are thought to be mitigated by the anti-inflammatory properties of exosomes—whereas ADE rats likely display only low-grade inflammation even after several deprivation cycles, it seems plausible that exosomes might not have been effective in the ADE model. This suggests that exosome treatment may not generalize across different genetic backgrounds.

The study by Ezquer et al. and the present multi-center investigation differ in the timing of the intervention, reflecting model-specific considerations. In the UChB rat model, animals voluntarily consume ethanol continuously for ~100 days, reaching stable intake levels of approximately 10–12 g/kg/day. This pattern allows interventions to be tested directly during a sustained phase of high ethanol consumption. In contrast, the alcohol deprivation effect (ADE) model relies on non-selected Wistar rats, which do not initially display compulsive alcohol use. Instead, repeated cycles of ethanol access and deprivation are required to induce escalation of intake and addiction-like behavior over time. Importantly, the first deprivation phase in the ADE paradigm occurs before animals have developed robust compulsive-like drinking. For this reason, interventions targeting relapse-like behavior are typically administered only after several deprivation cycles, when the phenotype of excessive and compulsive drinking has emerged [10–12]. The aim of our replication study was to evaluate whether exosomes—which were shown by Ezquer et al. to reduce ongoing ethanol consumption in UChB rats—would also attenuate relapse-like drinking in ADE rats with an established addiction-like phenotype. We did not assess exosomes prior to the first deprivation phase, since at that stage ADE rats do not yet exhibit the compulsive alcohol-seeking behavior that characterizes later phases of the model.

Despite a lacking impact of exosome treatment on actual relapse behavior, we observed lasting effects on ERO activity particularly within higher frequency ranges. In humans gamma oscillations are strongly modulated by alcohol [34]. In our previous studies, we showed that Wistar rats subjected to the long-term ADE procedure also display elevated gamma activity [19]. Notably, exosome-treated animals did not show this pathological gamma enhancement. Instead, they exhibited increased power in lower frequency bands, suggesting a shift toward a more 'healthy-like' oscillatory profile. However, this shift is much smaller than previously observed for other putative anti-relapse compounds (e.g. mGluR2 agonists) that also demonstrated behavioral efficacy on alcohol relapse [19]. Together, these findings provide functional proof that the intranasally applied exosomes indeed reached the brain and were active on prefrontal electrophysiological impairments in Wistar rats subjected to the long-term ADE procedure. However, this electrophysiological effect had no impact on relapse-like drinking in the ADE paradigm.

Our findings highlight the necessity for rigorous cross-model validation before advancing exosome therapy for alcohol addiction. The observed null results in our study suggest that the therapeutic effects reported by Ezquer et al. [7] may be model-specific rather than generally applicable. Future research should investigate the mechanistic underpinnings of these disparities, including potential interactions

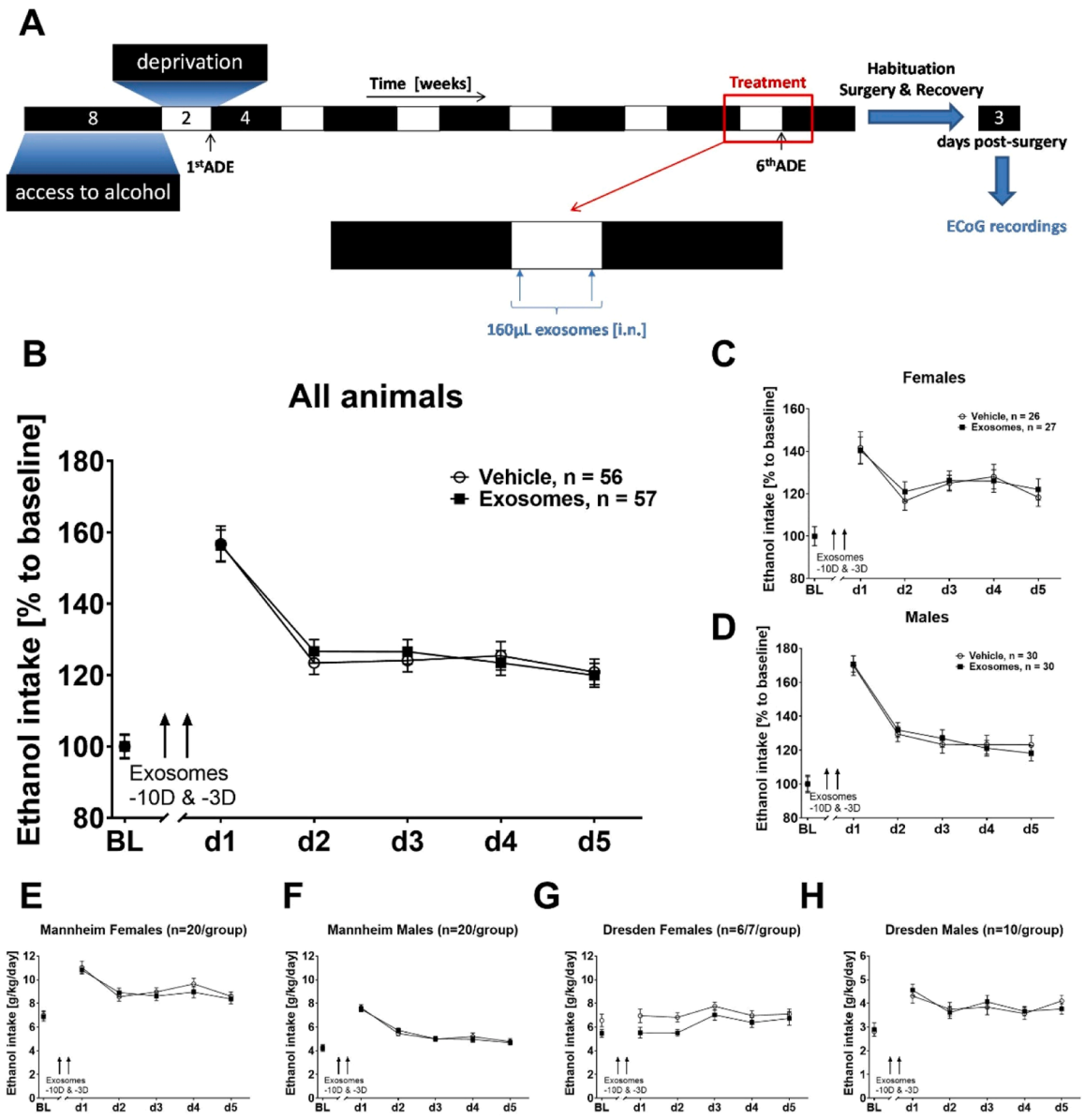

Fig. 2. Timeline and results of exosome treatment on relapse-like drinking (ADE) within the preRCT. (A) Numbers indicate weeks of every experimental phase. After habituation first alcohol consumption phase was initiated followed by an alcohol-free period (alcohol deprivation) and re-access (relapse/alcohol deprivation effect) to alcohol. Drug application phase is highlighted in red. Following the intervention, 20 male rats, 10 exosome-treated and 10 vehicle-treated, were operated for ECoG recordings **(B)** Effects of two exosome applications on day -10 and -3 before ADE on relapse-like drinking. Intake of total ethanol (calculated in g of pure alcohol per kg of body weight per day or then further calculated in % to baseline) is show before and after a deprivation period of two weeks. The last 3 days measurements of ethanol intake is given as baseline drinking—'BL'. Arrows indicate the administration of either vehicle, or of 7.5µg/160µl, intranasal exosomes. Panels **C** and **D** represent data obtained from males and females, respectively. This is further broken down to research site (Mannheim: **E&F** and Dresden: **G&H**). Note: The interim analyses at the two sites was done before site 3 (Erlangen) could start their experiments. This is why not data from Erlangen are shown here. Data are presented as means ± SEM.

between genetic predisposition, gut microbiome composition, and exosome-mediated neuroprotection. Additionally, further models such as the postdependent model—which induces physical dependence through chronic intermittent alcohol exposure—or the use of other genetically alcohol-preferring rat lines, such as Marchigian Sardinian alcohol-preferring rats should be tested, alternative dosing regimens, delivery methods, or exosome sources may warrant investigation to optimize therapeutic potential.

In conclusion, while the initial findings by Ezquer et al. [7] were promising, our present study does not support the efficacy of intranasal exosome treatment in reducing alcohol relapse behavior in Wistar rats. These findings underscore the importance of replication efforts in addiction biology and caution against premature clinical translation without broader validation. Publishing such null results not only contributes to a more balanced and accurate scientific record but also supports a more ethical and efficient research ecosystem.

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CRediT authorship contribution statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.addicn.2025.100234.

Data availability

raw data was uploaded to a Zenodo DOI: 10.5281/zenodo.15236345.

References

[1] N. Kafkafi, J. Agassi, E.J. Chesler, J.C. Crabbe, W.E. Crusio, D. Eilam, et al., Reproducibility and replicability of rodent phenotyping in preclinical studies, Neurosci. Biobehav. Rev. 87 (2018) 218–232.
[2] A. Bespalov, T. Steckler, B. Altevogt, E. Koustova, P. Skolnick, D. Deaver, et al., Failed trials for central nervous system disorders do not necessarily invalidate preclinical models and drug targets, Nat. Rev. Drug Discov. 15 (2016) 516.
[3] R. Spanagel, Ten Points to Improve Reproducibility and Translation of Animal Research, Front Behav. Neurosci. 16 (2022).
[4] M.W. Meinhardt, B. Gerlach, R. Spanagel, Good Practice Guideline for Preclinical Alcohol Research: The STRINGENCY Framework, Curr. Top Behav. Neurosci. (2024), https://doi.org/10.1007/7854_2024_484, 2024.
[5] M.W. Meinhardt, I. Skorodumov, J. Jeanblanc, F. Benvenuti, F.F. Hilal, E. Domi, et al., A new module in the drug development process: preclinical multi-center randomized controlled trial of R-ketamine on alcohol relapse, Neuropsychopharmacology (2025), https://doi.org/10.1038/S41386-025-02071-W, 2025.
[6] J. Gallardo, P. Berríos-Cárcamo, F. Ezquer, Mesenchymal stem cells as a promising therapy for alcohol use disorder, Int. Rev. Neurobiol. 178 (2024).
[7] F. Ezquer, M.E. Quintanilla, P. Morales, D. Santapau, M. Ezquer, M.J. Kogan, et al., Intranasal delivery of mesenchymal stem cell-derived exosomes reduces oxidative stress and markedly inhibits ethanol consumption and post-deprivation relapse drinking, Addic. Biol. (2018).
[8] M.E. Quintanilla, Y. Israel, A. Sapag, L. Tampier, The UChA and UChB rat lines: Metabolic and genetic differences influencing ethanol intake, Addic. Biol. 11 (2006) 310–323.
[9] N.I. Drude, L. Martinez-Gamboa, M. Danziger, A. Collazo, S. Kniffert, J. Wiebach, et al., Planning preclinical confirmatory multicenter trials to strengthen translation from basic to clinical research – a multi-stakeholder workshop report, Transl. Medicine Commun. 7 (1) (2022) 1–13, 20227.
[10] R. Spanagel, SM. Hölter, Long-term alcohol self-administration with repeated alcohol deprivation phases: an animal model of alcoholism? Alcohol Alcoholism 34 (1999) 231–243.
[11] V. Vengeliene, E. Celerier, L. Chaskiel, F. Penzo, R. Spanagel, Compulsive alcohol drinking in rodents, Addic. Biol. 14 (2009) 384–396.
[12] V. Vengeliene, A. Bilbao, R. Spanagel, The alcohol deprivation effect model for studying relapse behavior: A comparison between rats and mice, Alcohol 48 (2014) 313–320.
[13] R. Spanagel, Animal models of addiction, Dialogues Clin. Neurosci. 19 (2017) 247–258.
[14] J. Foo, M.W. Meinhardt, I. Skorodumov, R. Spanagel, Alcohol solution strength preference predicts compulsive-like drinking in rats, Alcohol Clin. Exp. Res. 46 (2022).
[15] S.M. Hölter, R. Spanagel, Effects of opiate antagonist treatment on the alcohol deprivation effect in long-term ethanol-experienced rats, Psychopharmacology (Berl) 145 (1999) 360–369.
[16] R. Spanagel, S.M. Hölter, K. Allingham, R. Landgraf, W. Zieglgänsberger, Acamprosate and alcohol: I. Effects on alcohol intake following alcohol deprivation in the rat, Eur. J. Pharmacol 305 (1996) 39–44.
[17] R. Spanagel, V. Vengeliene, B. Jandeleit, W.N. Fischer, K. Grindstaff, X. Zhang, et al., Acamprosate produces its anti-relapse effects via calcium, Neuropsychopharmacology 39 (2014) 783–791.
[18] B. Habelt, C. Wirth, D. Afanasenkau, L. Mihaylova, C. Winter, M. Arvaneh, et al., A multimodal neuroprosthetic interface to record, modulate and classify electrophysiological biomarkers relevant to neuropsychiatric disorders, Front Bioeng. Biotechnol. 9 (2021) 770274.
[19] B. Habelt, D. Afanasenkau, C. Schwarz, K. Domanegg, M. Kuchar, C. Werner, et al., Prefrontal electrophysiological biomarkers and mechanism-based drug effects in a rat model of alcohol addiction, Transl. Psychiatry 14 (2024) 486.
[20] O. George, C. Sanders, J. Freiling, E. Grigoryan, S. Vu, C.D. Allen, et al., Recruitment of medial prefrontal cortex neurons during alcohol withdrawal predicts cognitive impairment and excessive alcohol drinking, Proc. Natl. Acad. Sci. U S A 109 (2012) 18156–18161.
[21] O. George, GF. Koob, Control of craving by the prefrontal cortex, Proc. Natl. Acad. Sci. U S A 110 (2013) 4165.
[22] V. Vengeliene, R. Spanagel, mGlu2 mechanism-based interventions to treat alcohol relapse, Front Pharmacol. 13 (2022).
[23] S.M. Hölter, M. Engelmann, C. Kirschke, G. Liebsch, R. Landgraf, R. Spanagel, Long-term ethanol self-administration with repeated ethanol deprivation episodes changes ethanol drinking pattern and increases anxiety-related behaviour during ethanol deprivation in rats, Behav. Pharmacol. 9 (1998) 41–48.
[24] V. Vengeliene, D. Bachteler, W. Danysz, R. Spanagel, The role of the NMDA receptor in alcohol relapse: a pharmacological mapping study using the alcohol deprivation effect, Neuropharmacology 48 (2005) 822–829.
[25] V. Vengeliene, F. Leonardi-Essmann, W.H. Sommer, H.M. Marston, R. Spanagel, Glycine transporter-1 blockade leads to persistently reduced relapse-like alcohol drinking in rats, Biol. Psychiatry 68 (2010) 704–711.
[26] D. Afanasenkau, D. Kalinina, V. Lyakhovetskii, C. Tondera, O. Gorsky, S. Moosavi, et al., Rapid prototyping of soft bioelectronic implants for use as neuromuscular interfaces, Nat. Biomed. Eng. 4 (2020) 1010–1022.
[27] A. Delorme, S. Makeig, EEGLAB: An open source toolbox for analysis of single-trial EEG dynamics including independent component analysis, J. Neurosci. Methods 134 (2004) 9–21.
[28] C. Sanchis-Segura, JB. Becker, Why we should consider sex (and study sex differences) in addiction research, Addic. Biol. 21 (2016) 995–1006.
[29] J.R. Gerstner, JCP. Yin, Circadian rhythms and memory formation, Nat. Rev. Neurosci. 11 (2010) 577–588.
[30] M.E. Quintanilla, Y. Israel, A. Sapag, L. Tampier, The UChA and UChB rat lines: Metabolic and genetic differences influencing ethanol intake, Addic. Biol. 11 (2006) 310–323.
[31] F. Ezquer, M.E. Quintanilla, P. Morales, D. Santapau, J.M. Munita, F. Moya-Flores, et al., A dual treatment blocks alcohol binge-drinking relapse: Microbiota as a new player, Drug Alcohol Depend (2022) 236.
[32] F. Ezquer, M.E. Quintanilla, F. Moya-Flores, P. Morales, J.M. Munita, B. Olivares, et al., Innate gut microbiota predisposes to high alcohol consumption, Addic. Biol. 26 (2021).
[33] M. Díaz-Ubilla, A.I. Figueroa-Valdés, H.E. Tobar, M.E. Quintanilla, E. Díaz, P. Morales, et al., Gut microbiota-derived extracellular vesicles influence alcohol intake preferences in rats, J. Extracell Vesicles 14 (2025).
[34] J.U. Ramlakhan, M. Ma, R. Zomorrodi, D.M. Blumberger, Y. Noda, MS. Barr, The role of gamma oscillations in the pathophysiology of substance use disorders, J. Personalized Med. 11 (2021) 17. Page2020;11:17.
[35] R. Spanagel, P. Bach, T. Banaschewski, A. Beck, F. Bermpohl, R.E. Bernardi, et al., The ReCoDe addiction research consortium: losing and regaining control over drug intake—Findings and future perspectives, Addic. Biol. 29 (2024).