GENE- AND CELL-THERAPY ON THE ACQUISITION AND RELAPSE-LIKE BINGE DRINKING IN A MODEL OF ALCOHOLISM: TRANSLATIONAL OPTIONS.

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ABSTRACT

Studies reviewed show that *lentiviral gene therapy* directed either at inhibiting the synthesis of brain acetaldehyde generated from ethanol or at degrading brain acetaldehyde fully prevent ethanol intake by rats bred for their high alcohol preference. However, after animals have chronically consumed alcohol, the above gene therapy did not inhibit alcohol intake, indicating that in the chronic ethanol intake condition brain acetaldehyde is no longer the compound that generates the continued alcohol reinforcement. Oxidative stress and neuroinflammation generated by chronic ethanol intake are strongly associated with the perpetuation of alcohol consumption and alcohol relapse "binge drinking". Mesenchymal stem cells, referred *to as "guardians of inflammation"*, release anti-inflammatory cytokines and antioxidant products. The intravenous delivery of *human mesenchymal stem cell*s or the intranasal administration of *mesenchymal stem cell-generated exosomes* reverses both (i) alcohol-induced neuro-inflammation and (ii) oxidative stress, and greatly iii) inhibits (80 to 90%) chronic alcohol intake and relapse binge-drinking. The therapeutic effect of mesenchymal stem cells is mediated by increased levels of the brain GLT-1 glutamate transporter, indicating that glutamate signaling is pivotal for alcohol relapse. *Human mesenchymal stem cells* and the products released by these cells may have translational value in the treatment of alcohol-use disorders.

Gene- and cell-therapy have provided valuable tools to unravel the mechanisms that (i) lead to alcohol reinforcement; (ii) perpetuate alcohol intake and (iii) are responsible for relapse binge-drinking. These studies also provide translational leads that will likely translate into human use. The accomplishments were supported by the existence of a few rat lines/strains bred to prefer drinking 10% ethanol solutions to drinking water. Three of such lines are available: the UChB in Chile (1, 2), the P, HAD strains in the Unites States (3) and sP rat line in Italy (4). The voluntary ethanol self-administration model avoids the need to externally administer ethanol, which often generates blood alcohol levels that exist only in severe alcoholism. Studies presented in this review were conducted in the UChB rat line, which was selectively bred as alcohol-preferring animals for 80 to 90 generations over 70 years. Upon a long-term alcohol (10%) availability, these animals consume 4-fold more of the ethanol solution than water, ingesting up to 12 g ethanol/Kg body weight/day (*vide infra*), which translates as one liter of pure alcohol consumed daily for a 70 kg individual. Consistent with scientific standards, these animals are available to investigators in other countries.

Initial reinforcing effects of ethanol: Acetaldehyde and gene transduction.

Most of the early preclinical studies in the alcoholism field addressed the mechanisms that lead to an initial alcohol intake by rats. Research by several groups have suggested that ethanol itself is not the initial reinforcing molecule, but rather ethanol is a prodrug (5), where the rewarding effect would be generated by acetaldehyde, the ethanol metabolite generated in the brain. Due to the absence of alcohol dehydrogenase in the brain, the generation of acetaldehyde in the brain is mainly carried out by catalase (6) (Figure 1). Data derived from three different experimental conditions support the view that acetaldehyde generated by catalase is reinforcing (i) catalase inhibition by 4-aminotriazole reduces initial ethanol intake (7-9), (ii) administration of the drug

penicillamine, which binds acetaldehyde, reduces early voluntary ethanol intake (10, 11) and (iii) rats self-administer acetaldehyde into a dopaminergic brain area (the posterior ventral tegmental area, VTA) at concentrations that is three-order of magnitude lower than those needed for ethanol self- administration (12). A full review of these drug studies can be found in Acquas et al (13). Since drug specificity is generally questioned, and animals will also self-administer many drugs of abuse that are not generated endogenously, it was felt that a more specific (non-drug-related) gene-based inhibition of brain acetaldehyde generation was needed to support the view that acetaldehyde was the reinforcing molecule generated from ethanol.

Figure 2 shows that the administration of a lentiviral vector coding for an anti-catalase shRNA into the brain ventral tegmental area (VTA) completely inhibited the acquisition of voluntary alcohol intake by rats (14). Since all drugs of abuse increase the release of dopamine in nucleus accumbens (15), it was expected that the anti-catalase shRNA coding vector would also inhibit dopamine release induced by the acute administration of ethanol. This was indeed demonstrated as the anti-catalase shRNA fully prevented the increase in (VTA-mediated) dopamine release induced by an acute oral ethanol dose (1 g/kg) in nucleus accumbens (14). The release of dopamine induced by amphetamine was however not affected by anti-catalase shRNA, thus indicating that its inhibitory effect on dopamine release is specific for ethanol (14).

Figure 3 shows that VTA transduction of a lentiviral vector coding for aldehyde dehydrogenase 2 (ALDH2), known for its high affinity for oxidizing acetaldehyde into acetate, virtually abolished ($p<0.001$) the acquisition of voluntary alcohol intake by rats (16). An additional question was thus posed: if increasing the degradation of acetaldehyde inhibits alcohol intake; will conversely, ethanol intake be enhanced by *increasing the generation of acetaldehyde* in the VTA? This was

addressed by transducing into the VTA a lentiviral vector coding for liver alcohol dehydrogenase (ADH), which oxidizes ethanol into acetaldehyde. Figure 4 shows that transduction of liver ADH into the VTA markedly increased ($p<0.001$) alcohol intake (to avoid a ceiling effect the ethanol available to the animals was 5% rather than 10%). Overall, these gene therapy studies fully support the view that brain-generated acetaldehyde is the reinforcing agent that promotes early ethanol intake.

The above studies suggested that inhibiting brain acetaldehyde generation or increasing its degradation might be a tool to inhibit chronic alcohol consumption once a high ethanol consumption has been attained. As will be seen, this was (surprisingly) not the case, thus opening an important new lead of investigation. As seen in Figure 5, following chronic alcohol consumption for 60 to 80 days, the transduction of the lentiviral vectors coding either for the shRNA anti-catalase or the ALDH2 enzyme did not reduce chronic alcohol intake. Thus, *chronic alcohol intake no longer depends on brain acetaldehyde generation or its degradation*. This view was supported by studies by Peana et al (11) who showed that neither 4-aminotriazole (inhibitor of catalase) nor penicillamine (which binds acetaldehyde), inhibited *chronic* alcohol self-administration, although these were effective in inhibiting the initial ethanol intake. Thus, the new question was to define the mechanism(s) that keep *chronic* ethanol intake (*vide infra*). Noteworthy, these animals do not develop a withdrawal reaction, and thus negative reinforcement is not the mechanism that keeps chronic ethanol intake unabated (Quintanilla et al 2012).

A gene therapy approach to generate an aversion to alcohol.

Most of ethanol consumed is oxidized in the liver by ADH, generating acetaldehyde, which does not readily cross the blood brain barrier (18). Acetaldehyde generated does not accumulate nor is found in high concentrations in the circulation since it is readily converted into acetate by liver ALDH2, a dehydrogenase with a high-affinity for acetaldehyde (<0.2 μM) present in virtually all tissues (19). However, an important proportion (20-25%) of individuals in East Asia (Japan, Korea, China) present a dominant negative mutation of the *ALDH2* gene (E487K) which renders the enzyme virtually inactive, thus allowing increases in blood acetaldehyde to levels which generate nausea, hypotension and tachycardia. These reactions deter subsequent drinking in individuals carrying the *ALDH2* gene (E487K), such that Individuals who are heterozygous for the E487K mutation are 70 to 85% protected against alcoholism (20-21). Such a protection against alcoholism suggested that inhibiting hepatic ALDH2 synthesis by treatment with a liver directed anti ALDH2 shRNA or an antisense oligonucleotide would generate an aversion to alcohol in animals after these have become chronic ethanol drinkers. Figure 6 shows that rats that had consumed alcohol chronically for 2 months, which were deprived of ethanol for 3 days and were subsequently allowed a 10% ethanol re-access for only 60 minutes consume binge amounts of 1.0 to 1.2 g ethanol/kg body weight in 60 minutes (Control AdV). Animals that intravenously received an adenoviral vector coding for an antisense mRNA against the *Aldh2* transcript, which lowered ALDH2 activity by 90% ($p<0.02$), consumed 50% less alcohol ($p<0.001$) than control animals, an effect that was maintained over one month (22). Thus, animals treated with the anti ALDH2 antisense developed an alcohol-aversion phenotype, akin to that seen in Asians carrying the E487K mutation. Similar inhibition of alcohol intake was reported for animals that were subcutaneously implanted a minipump that delivered an anti-*Aldh2* antisense oligonucleotide 23) or in animals that were administered an adenoviral vector coding for a ribozyme against *Aldh2* mRNA (24).

These studies will likely be continued to translational fruition by the (cautious) use of CRISPR/Cas9 or shRNA to reduce only liver ALDH2 synthesis without the use of viral vectors. Several studies in the gene therapy field have shown that oligonucleotides/genes can be specifically delivered to hepatocytes if attached to a galactose moiety or mixed with a lactosylated carrier, which are taken up by hepatocytes via the asialoglycoprotein receptor (25). A complete inhibition of ALDH2 gene expression in other tissues should be avoided as alcoholics who carry the inactivating *ALDH2* (E487K) mutation display elevated rates of upper gastrointestinal cancers (26). Present view in the alcohol-use disorder treatment field is that substantial reductions in alcohol intake which do not reach (or aim to) a full abstinence have a positive value (27,28).

The alcohol induced oxidative stress-neuroinflammation cycle: basis for cell therapy

A number of reports (29, 30) have shown that chronic ethanol intake or its administration lead to brain (a) neuroinflammation and (b) oxidative stress. Several mechanisms play a role in inducing these changes (i) ethanol in the gut is oxidized by intestinal bacteria generating acetaldehyde, which weakens the intestinal tight junctions, allowing the passage of bacterial lipopolysaccharide (31) that activates the generation of TNF-α by liver macrophages, and further via brain TNF–α receptors (32) induces the synthesis of brain proinflammatory cytokines and the generation of oxygen radicals via NADPH oxidase. Two additional ethanol-related mechanisms may activate the generation of brain free radicals; first the monoamine oxidase production of hydrogen peroxide generated in the oxidation of dopamine released by ethanol in nucleus accumbens (33); secondly, the generation of free-radicals by CYP2E1, a cytochrome, which is markedly increased in the brain following chronic ethanol intake (34).

Figure 7 shows that rats that have consumed 10% ethanol for 11 weeks, reaching an ethanol intake of 9-10 g ethanol/kg/day, display both a marked increase in hippocampal oxidative stress as shown by an elevated oxidized glutathione/reduced glutathione (GSSG/GSH) ratio and marked neuroinflammation as shown by marked increases in the length and thickness of astrocyte process marked by glial fibrillary acidic protein (GFAP) immunoreactivity (35), Noteworthy, oxidative stress and neuroinflammation are tightly linked since reactive oxygen species activate the NF-kB generation of proinflammatory cytokines, including TNF-α which in turn uncouples mitochondria generating superoxide radicals and further hydrogen peroxide (36),. Thus, oxidative stress and neuroinflammation self-perpetuate each other in a vicious cycle manner. It has been reported that a single i.p. dose of lipopolysaccharide administered to rats generates neuroinflammation lasting over 9 months (32).

Pro-inflammatory conditions increase voluntary alcohol consumption in rodents and humans (37; 38). Research has indicated that alcohol-induced neuroinflammation remains up-regulated for long periods even after discontinuation of alcohol consumption, in line with the existence of a potent mechanism of auto-perpetuation of neuroinflammation. This phenomenon is associated with a marked increase in the risk of relapse in abstinent patients (39).

Alcohol relapse in animals fed alcohol chronically is causally associated with the existence of high levels of extracellular glutamate since the administration of the antibiotic ceftriaxone, which increases the levels of the astrocyte Na-glutamate transporter-1 (GLT-1) and removes glutamate from the tripartite synapse extracellular space markedly reduces alcohol relapse (40). Increases in GLT-1 transporter levels are required to reduce relapse since the glutamate transporter GLT-1 is inhibited by oxidative stress (41) and by 4-hydroxynoenal, a lipid aldehyde generated in oxidative

198 stress conditions (42). Considering the marked potentiation between oxidative stress and
199 neuroinflammation indicated above, the increases of both conditions induced by chronic ethanol
200 intake is expected to reduce GLT-1 activity and to increase extracellular glutamate.
201
202 Cell therapy based on mesenchymal stem cells (MSCs) is emerging as a preclinical option for
203 various diseases in which the immune system is over-activated. Mesenchymal stem cells have
204 been referred to as the "guardians of inflammation" (43). Recent studies in a well-validated animal
205 model of high-alcohol intake (UChB) showed that the intra-cerebroventricular administration of
206 activated rat MSCs (44) or human MSCs (45) reduced up to 85% chronic alcohol intake and
207 relapse-like drinking in high drinker rats. Clearly, the intra-cerebroventricular administration is not
208 a preferred route for the treatment of a chronic disease such alcoholism. Two new advances have
209 dealt with this shortcoming. We have recently shown that MSCs extracted from human
210 liposuction fat and grown in 3D-hanging droplets (spheroids) attain a volume equivalent to that of
211 human lymphocytes, which if injected intravenous to the rat are able to reach the brain (46). A
212 single intravenous administration of MSC spherocytes (1x 10^6 cells) to rats that had consumed 10%
213 and 20% ethanol for a total of 12 weeks reduced their alcohol intake by 93% within 48 hours, an
214 effect (-75% inhibition) that remained for at least 3 weeks. (Figure 8A left). The reduction in
215 ethanol (solutions) intake induced by the MSC administration was compensated by increases in
216 water intake (Figure 8B). Animals were further deprived of alcohol for 2-weeks and subsequently
217 offered ethanol (10 % and 20%) solutions for only 60 minutes. In such a short period, control
218 animals (that had ingested alcohol for 12 weeks plus 2 weeks of ethanol deprivation and received
219 only vehicle rather than MSCs) consumed binge amounts of alcohol that led their blood alcohol
220 concentration (BAC) to 100 mg ethanol/dl. Animals that received the single dose of MSC
221 spheroids 5 weeks prior to the ethanol re-access reduced their intake by 80% (Figure 8A right),

reaching BACs of only 19 mg ethanol/dl (Figure 8C) which are within the levels that in humans would be considered achieved after social drinking.

Three major mechanism-related questions were addressed in the above studies (46). Did the intravenous MSC-spheroid administration: (i) inhibited ethanol-induced neuroinflammation (ii)reduced brain oxidative stress and (iii) increased the levels of the glutamate transporter GLT-1.

(i) Chronic alcohol intake markedly increased the length and thickness of astrocytic processes as seen by GFAP, indicative of neuro-inflammation. (Figure 8D-center versus 8D-left). Administration of MSCs reversed the neuro-inflammation (Figs 8D center vs 8D right; Figs 8E and 8F). A likely mechanism involved in the MSC-spheroid mediated inhibition of neuroinflammation is the 3D-activation of anti-inflammatory IL-10 and TSG-6 cytokine production (46);

(ii) Mesenchymal stem cells administration also fully abolished the alcohol- induced oxidative stress as determined by the normalization of the GSSG/GSH ratio (Figure 8G);

(iii) Figure 9 shows that the administration of MSC to ethanol fed animals markedly increased the GLT-1 levels both in prefrontal cortex and nucleus accumbens (46) which likely constitutes the mechanism by which chronic ethanol intake and relapse are inhibited by MSC administration (46)

The protracted effects observed after the administration of intact human MSCs to a rat is in line with the low expression of the major histocompatibility complex (MHC) in human MSCs (47), which avoids a T-lymphocyte-induced recognition and elimination. Thus, the spheroids constitute a long-acting “pump” which secretes anti-inflammatory factors into the bloodstream. However,

the final fate of live MSCs delivered in the circulation is not known, and for a safe translational delivery we determined if intranasally administered MSC-derived exosomes (small nanovesicles shed *in vitro* following cytokine activation of MSCs) would also reproduce the effect of live MSC "pumps"; even if with a shorter effect. In studies reported by Ezquer et al (48) exosomes were generated from MSCs activated by incubation with proinflammatory TNF-α and IFNγ; obtaining a conditioned medium that was ultracentrifuged to precipitate the exosomes and washed thoroughly to remove the proinflammatory agents. Exosomes tagged with PKH26 a fluorescent dye showed that exosomes administered to rats by intranasal instillation reach the brain within 24 hours (48).

Figure 10 A shows that following 3 intranasal doses of exosomes administered at weekly intervals (each dose generated by 1 x 10^6 MSCs) to rats that had chronically consumed ethanol for 12 to 13 weeks, ethanol intake was inhibited by 80 to 85%. The reduction of ethanol (solutions) intake induced by intranasal MSC-exosomes was compensatory by increases in water intake, thus maintaining animal's water homeostasis (Figure 10B). Intranasal exosome administration inhibited by 50% the ethanol relapse drinking that followed a 2-week ethanol deprivation and a short period of ethanol re-access (Figure 10C), which prevented "binge drinking" by reducing of BAC from 110 mg ethanol/dl to 58 mg ethanol/dl (Figure 10D). Exosomes also reversed alcohol induced neuroinflammation and oxidative stress and markedly increasedGLT-1 gene expression in nucleus accumbens (48).

Overall, the studies reviewed show that lentiviral gene therapy aimed at inhibiting the generation of brain acetaldehyde derived from ethanol or at degrading brain acetaldehyde nearly completely prevented the early ethanol ingestion by rats bred for their high alcohol preference. After alcohol-

naive animals have chronically consume large amounts of alcohol for prolonged times, the above gene therapy approaches are not effective indicating that brain acetaldehyde was no longer the reinforcing agent supporting chronic alcohol intake. However, *mesenchymal stem cell therapy* approaches, along with the noninvasive intranasal delivery of activated mesenchymal stem cell products, were most effective in markedly inhibiting both chronic alcohol intake and relapse binge-drinking; studies that indicated that the concerted inhibition of brain neuroinflammation and oxidative stress by mesenchymal stem cells constitutes the most likely mechanism for their therapeutic action. The latter, likely by counteracting an oxidative stress-impaired GLT-1 glutamate transport ability, both by the strong antioxidant action of mesenchymal stem cells and by activating GLT-1 gene expression. The noninvasive intranasal delivery of active exosomes released by human mesenchymal stem cells may have translational value in the treatment of alcohol-use disorders.

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LEGENDS TO FIGURES

Figure 1. Ethanol is metabolized into acetaldehyde in brain mainly by catalase and to a minor extent by cytochrome P450 (CYP2E1). The enzyme aldehyde dehydrogenase (ALDH), found in the cell's mitochondria degrades acetaldehyde to acetate (re-drawn from Zimatkin et al, ref 6)

Figure 2. A single dose of an anticatalase-lentiviral vector injected into the brain ventral tegmental area (VTA) of ethanol naïve rats led to a marked and long-lasting inhibition of their voluntary ethanol intake. Naïve female UChB rats anesthetized with a mixture of air and isoflurane were stereotaxically injected into the left VTA with either a single dose of 1 µl of an anticatalase lentiviral vector (8×10^4 viral particles) encoding a shRNA against catalase (anticatalase-Lenti) or an empty lentiviral vector and after four days of water intake the animals were allowed continuous (24 hours/day) free-choice access to ethanol solution (10% v/v) or water for sixty days. Ethanol naïve rats injected with a single dose of anticatalase-Lenti displayed a marked ($p < 0.001$) inhibition of the initial voluntary alcohol intake for the two months tested, compared to control empty lentiviral vector-treated rats. Figure redrawn. Complete methodologies and statistical analysis are found in Karahanian et al ref 14.

Figure 3. A single dose of a lentiviral vector coding for aldehyde dehydrogenase-2 (ALDH2-Lenti) injected into the brain VTA of ethanol naïve rats led to a marked and long-lasting inhibition of ethanol intake. Ethanol **n**aïve female rats anesthetized with a mixture of air and isoflurane were

stereotaxically injected into the left VTA with either a single dose of 1 µl of a lentiviral vector encoding for the rat ALDH2 (8×10^4 viral particles) (ALDH-Lenti) or an empty lentiviral vector (control-Lenti) and after four days of water intake the animals were allowed continuous (24 hours/day) free-choice access to ethanol solution (10% v/v) or water for forty five days. Ethanol naïve rats, which had been injected with a single dose of ALDH2-Lenti into the VTA displayed a significantly ($p < 0.001$) inhibition of the initiation of their voluntary alcohol intake, during the forty-five days tested, compared to control empty lentivirus-treated rats (control-Lenti). Figure redrawn Complete methodologies and statistical analysis are found in Karahanian et al ref 14.

Figure 4. A single dose of a lentiviral vector encoding for liver alcohol dehydrogenase (ADH-Lenti) injected into the brain VTA of ethanol naïve rats increases the voluntary ethanol intake in rats. Naive female UChB rats anesthetized with a mixture of air and isoflurane were injected into the left VTA either a single dose of 1 µl of a lentiviral vector (8×10^4 viral particles) encoding for ADH (ALDH-Lenti) or an empty Lentiviral vector (control-Lenti). After four days the animals were allowed continuous (24 hours/day) free-choice access to ethanol solution (5% v/v) or water for thirty days. Ethanol naïve rats, which had been injected with a single dose of lentiviral vector encoding alcohol dehydrogenase (Lenti-ADH) displayed a significantly ($p < 0.001$) higher voluntary alcohol intake, compared to control empty lentivirus-treated rats. Figure redrawn. A complete statistical analysis is found in Karahanian et al ref 14.

Figure 5. A **single dose of an shRNA anticatalase-coding lentiviral vector or a lentiviral vector encoding for ALDH2 injected into the brain VTA of rats that had been drinking ethanol for two or three months did not reduce the chronic ethanol intake.** Rats that had been under continuously

(24-hours) fre choice access between ethanol solution (10%) or water, for sixty or eighty days were anesthetized with a mixture of air and isoflurane and stereotaxically injected into the left VTA (B-5.6, L-0.5, V-7.4) with either **(A)** a single dose of 1 µl of an anticatalase lentiviral vector (8×10^4 viral particles) encoding for a shRNA against catalase (anticatalase-Lenti) or a empty lentivirus (n = 10 per group) or **(B)** a single dose of 1 µl of a lentiviral vector encoding for the rat ALDH2 (8×10^4 viral particles) (ALDH-Lenti) or an empty lentivirus (control-Lenti). Thereafter, the animals were transferred to their home cage and allowed 24 hours of continuous access to ethanol solution (10%) and water, for eighteen or eleven days. Results showed that neither the anticatalase-Lenti **(A)** nor the ALDH-Lenti **(B)** administration into the VTA of chronic ethanol drinking rats inhibited their voluntary alcohol intake versus that of the respective control groups (Redrawn from Quintanilla et al ref 17 and Tampier et al ref 9).

Figure 6. A **single dose of an adenoviral vector coding for an anti-Aldh2 antisense (AdV-AS) injected into the tail vein of rats that had been drinking ethanol for two months reduced ethanol intake by 50%.** Rats were allowed unrestricted access to 10% ethanol and water for 60 days, time at which their voluntary ethanol consumption was 7 to 8 g ethanol/kg/day. Subsequently, access to ethanol was discontinued and animals received a single intravenous injection of anti-Aldh2 antisense (AdV-AS) adenovirus or AdV-control (1×10^{12} vp/kg). After a 3-day ethanol-withdrawal period animals were allowed access to 10% (v/v) ethanol for only 1 hour each day, while water was continuously available. Results show that animals that received AdV-AS consumed 50% less ethanol than control animals ($p < 0.001$). Figure redrawn. Complete methodology and statistical analysis are found in Ocaranza et al ref 22.

Figure 7. Chronic ethanol consumption induced marked oxidative stress, determined by the ratio of hippocampal oxidized/reduced glutathione), and neuroinflammation determined by astrocyte immunofluorescence. (A) Rats under free-choice intake of ethanol (10% v/v) and water for 11 weeks reached an ethanol intake of 9-11 g ethanol /kg body weight/day; **(B)** Chronic ethanol intake induced an increased ($p<0.001$) hippocampus GSSG/GSH ratio compared with that in rats consuming only water and **(C)** Micrographs of glial fibrillary acidic protein (GFAP, green marker) counterstained with DAPI (blue, nuclear marker show that these chronic ethanol intake induced increased ($p<0.01$) immunoreactivity of GFAP in hippocampal astrocytes compared with rats consuming only water. Figure redrawm. A complete statistical analysis is found in Ezquer et al ref 45.

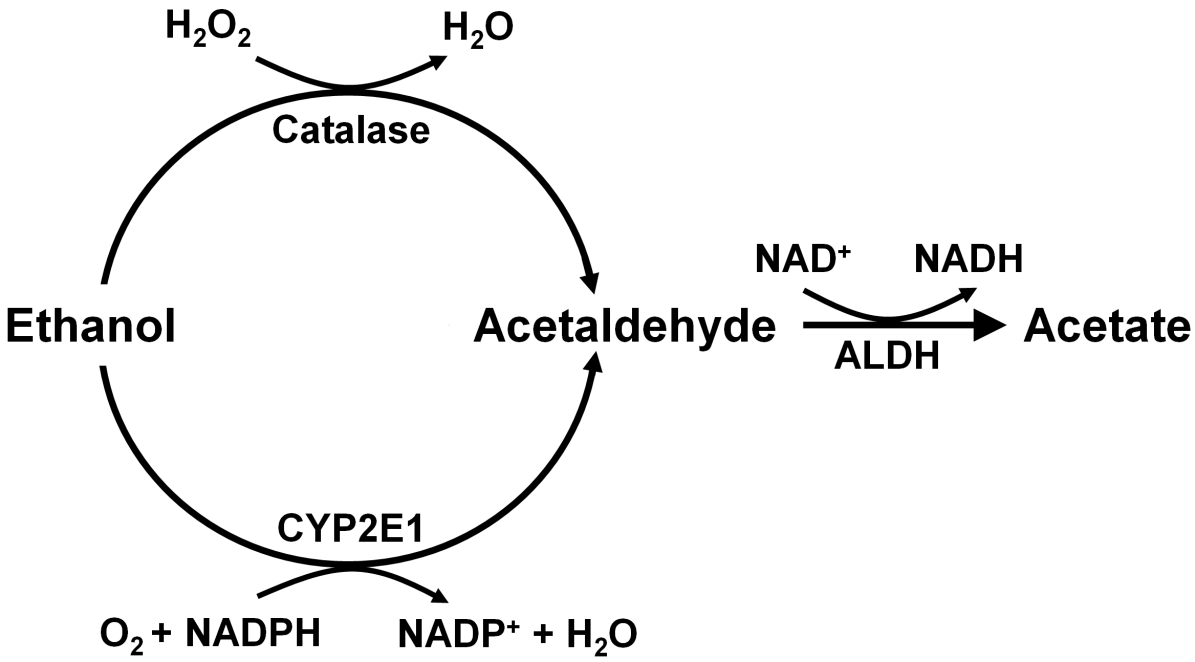
Figure 8. Intravenous administration of mesenchymal stem cells 3D-cultured (MSC)-spheroids inhibits chronic ethanol intake and relapse-like binge drinking in the ethanol post-deprivation condition (ADE), and normalizes astrocyte (GFAP) processes and oxidative stress (GSSG/GSH ratio) (A left) A single dose of 1×10^6 MSC-spheroids injected into the tail vein of rats markedly inhibited ($p<0.001$) voluntary ethanol intake of animals that had consumed ethanol for 12 weeks versus rats treated with vehicle. **(A right)** MSC-spheroids reduced ($p<0.01$) the relapse-like drinking ethanol intake (black bar) of the same animals, after an ethanol deprivation and a 60-min ethanol re-uptake period versus vehicle treated rats (white bar). **(B)** A single dose of MSC-spheroids that had resulted in a marked inhibition of ethanol intake (showed in Figure 8A-left), induced, in the same animals, a significant ($p<0.001$) increase in water intake versus vehicle treated rats. **(C)** The MSC-spheroids reduction of ethanol intake during the 60-min ethanol re-access **(showed in A right)** resulted in significant ($p<0.001$) lower blood alcohol levels versus that those of vehicle trated rats. **(D)** Confocal microscopy microphotographs of GFAP immunoreactivity

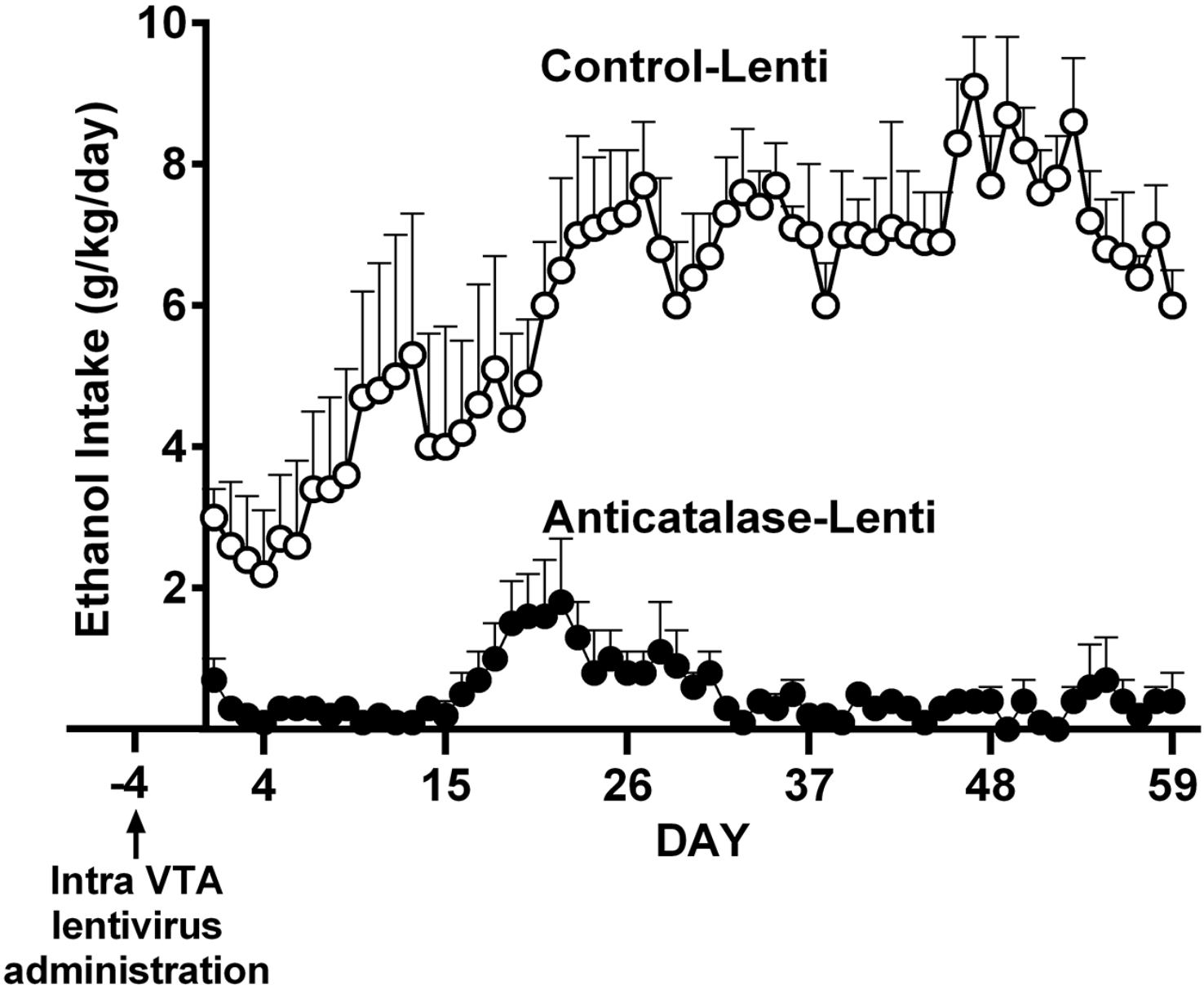
evaluated in hippocampal astrocytes of animals intravenously injected with 1×10^6 MSC-spheroid or vehicle, after the 60-minute ethanol re-access. Chronic ethanol drinking rats treated with vehicle displayed marked increases in the length and thickness of astrocyte processes (**E, gray bar)** versus rats drinking only water (**E, white bar**). Intravenous administration of a single dose of 1×10^6 MSC-spheroids, to chronic ethanol drinking rats normalized the length **(E, black bar)** and thickness **(F, black bar)** of astrocytic process. **(F)** Chronic ethanol drinking rats treated with vehicle displayed an increased the GSSG /GSH ratio (grey bar) versus ethanol naïve rats drinking only water (white bar). Administration of MSC-spheroids to chronic ethanol drinking rats normalized the GSSG/GSH ratio (black bar). A complete statistical analysis are found in Ezquer et al ref 46.

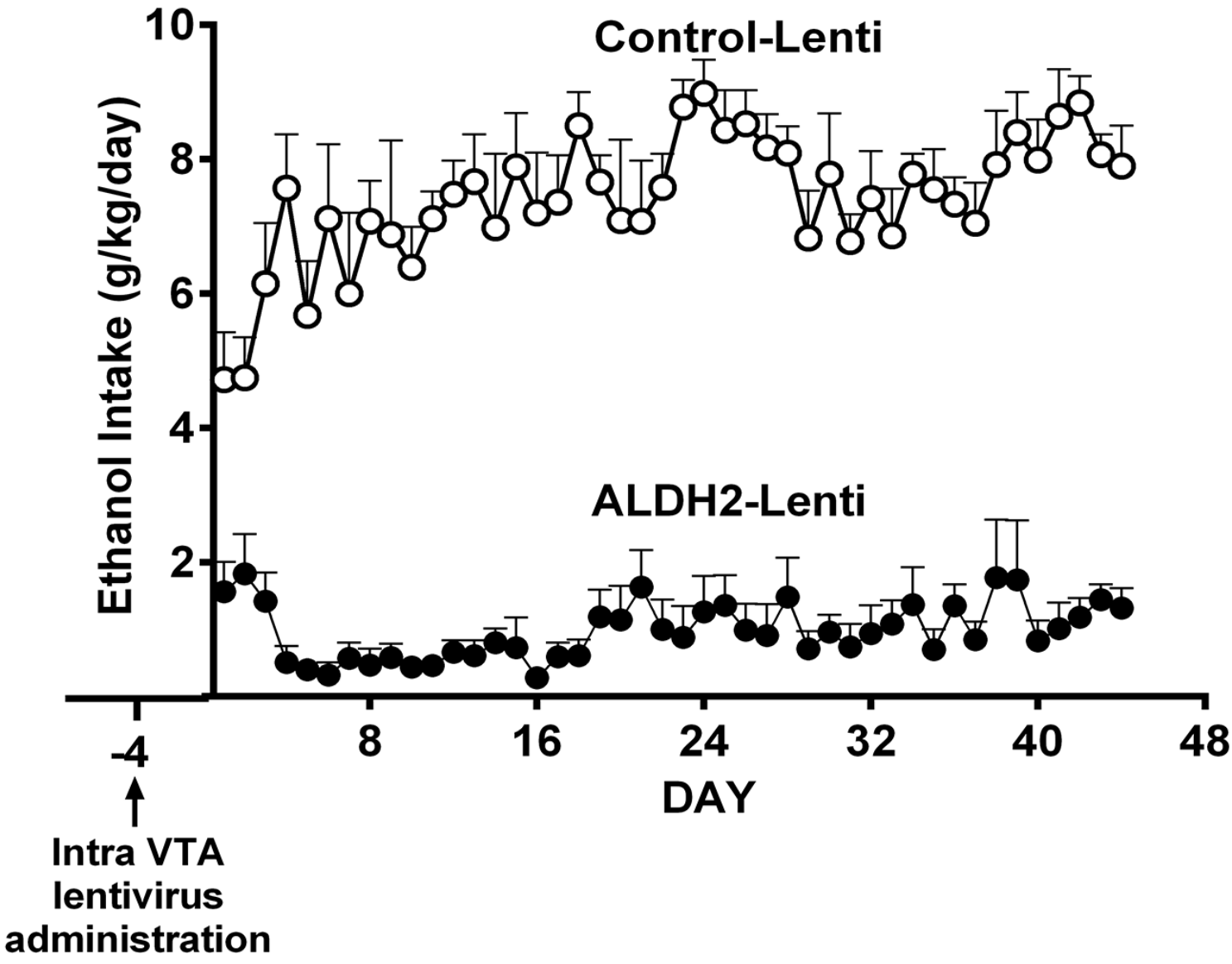
Figure 9. Intravenous administration of MSC-spheroids increases brain glutamate transporter GLT-1 levels. GLT-1 level in prefrontal cortex and nucleus accumbens of rats that had consumed ethanol for 12 weeks and were intravenously injected with a single dose of 1×10^6 MSC-spheroids or vehicle. GLT-1 levels were evaluated by Western blot analysis six weeks after MSC-spheroid or vehicle administration. After the MSC-spheroid administration the animals remained for four weeks in the ethanol and water free-choice condition, followed by a 2-week ethanol deprivation period and a 60-minute ethanol re-access. Animals consuming only water were used as untreated controls. Data are presented as percentage ratios of GLT-1/β-actin, relative to control levels. A complete statistical analysis snd methodologies are found in Ezquer et al ref 46.

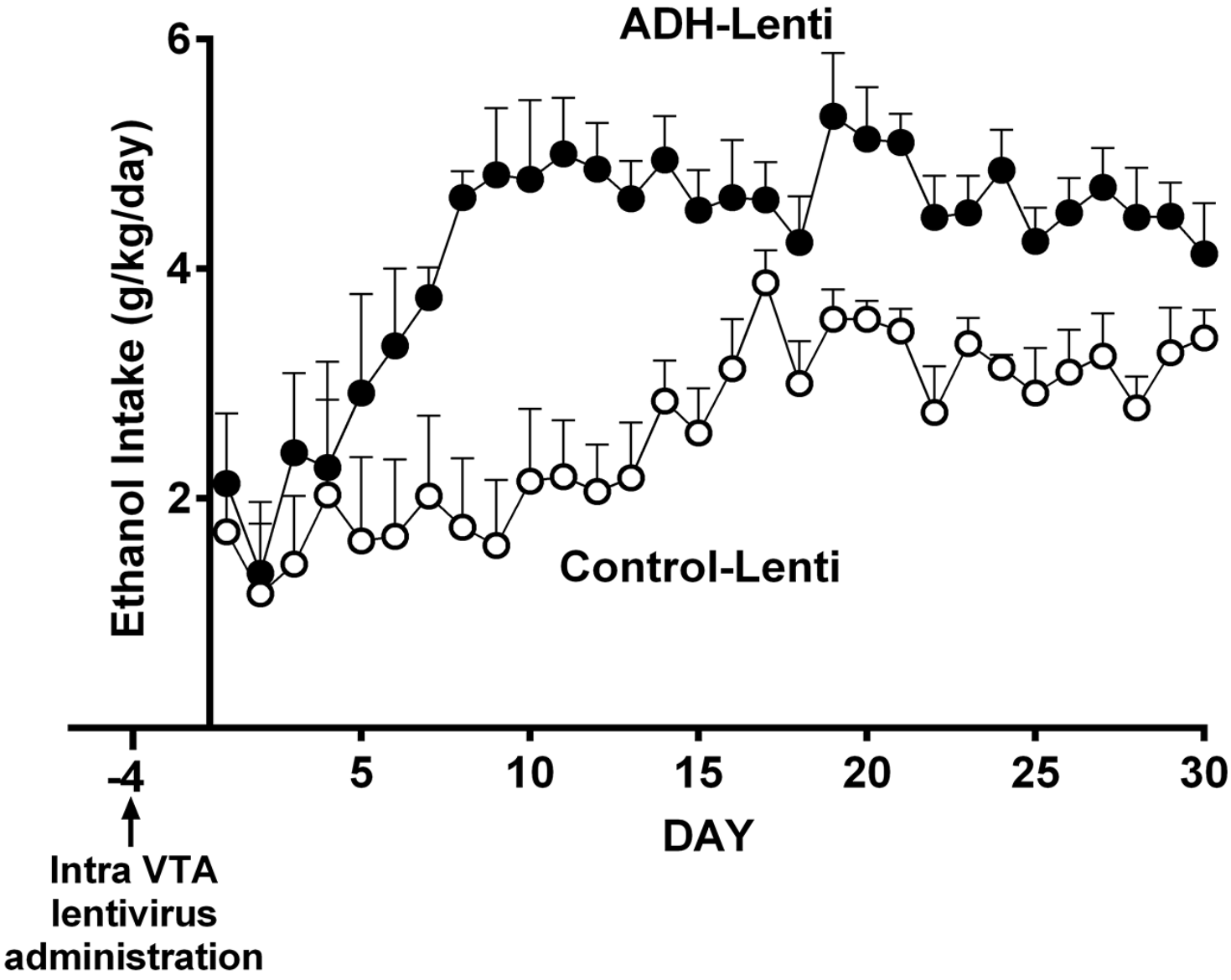
Figure 10. Intranasal administration of MSC-derived exosomes inhibits chronic ethanol intake and relapse-like ethanol intake in the ethanol post-deprivation and re-access condition. (A) Voluntary ethanol intake of animals that had consumed ethanol for 13 weeks a received three

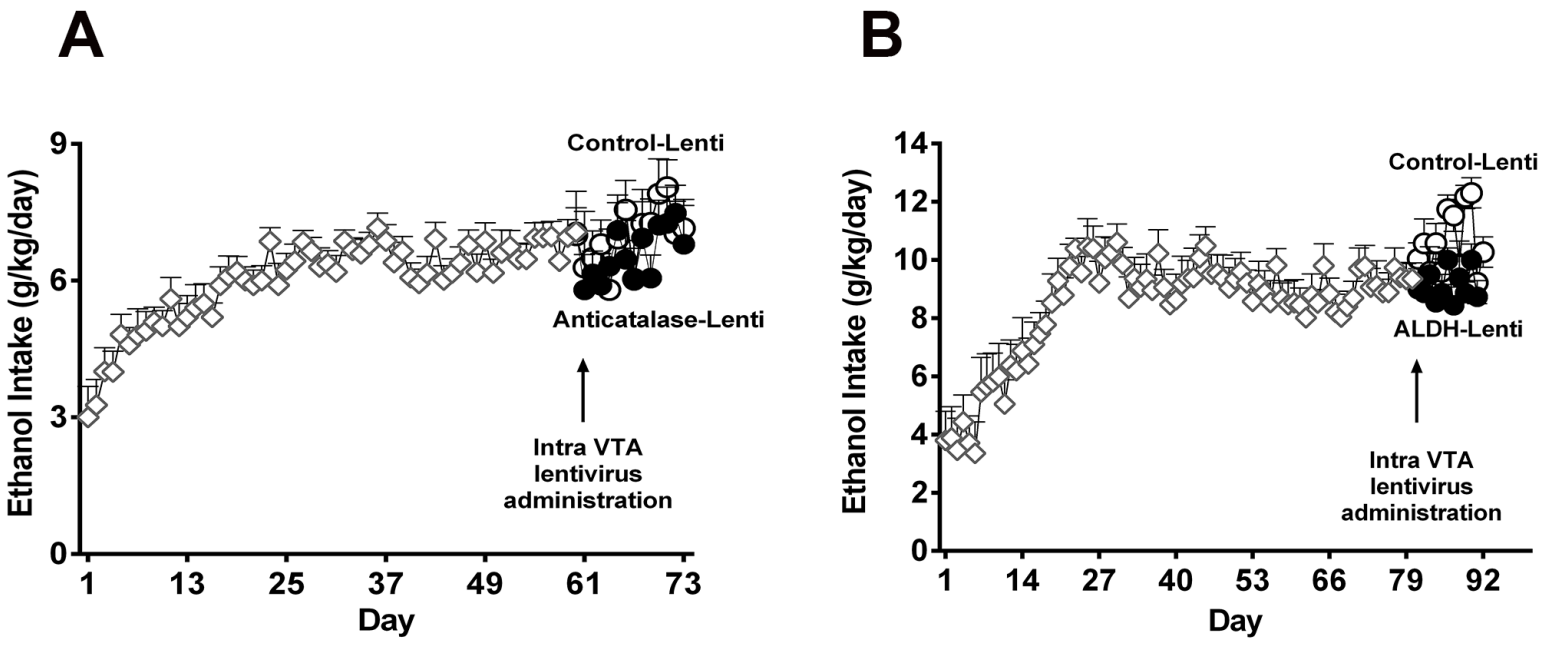
intranasal doses of exosomes derived from activated human MSCs (1.5×10^9 exosomes derived from 1×10^6 MSC each dose) or vehicle. Ethanol intake is expressed as g of ethanol consumed/kg body weight/day. **(B)** Voluntary water intake of animals shown above in (A). Data are expressed as ml of water consumed /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 from tail vein samples were determined immediately after the 60-minute relapse-like ethanol consumption A complete statistical analysis snd methodologies are found in Ezquer et al ref 48.

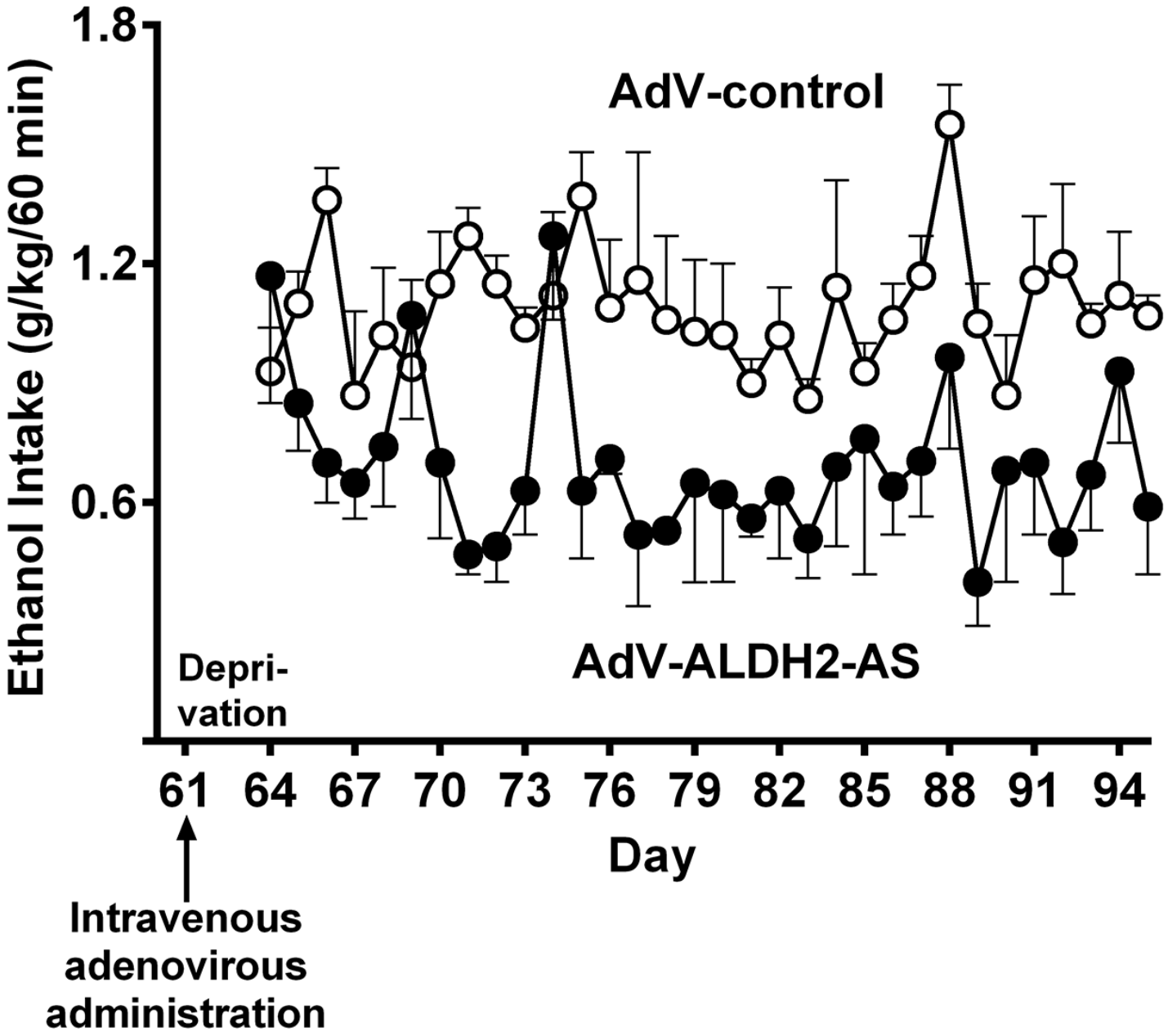

H2O2
H2O
Catalase
NAD+
NADH
Ethanol
Acetaldehyde
Acetate
ALDH
CYP2E1
O2 + NADPH
NADP+ + H2O

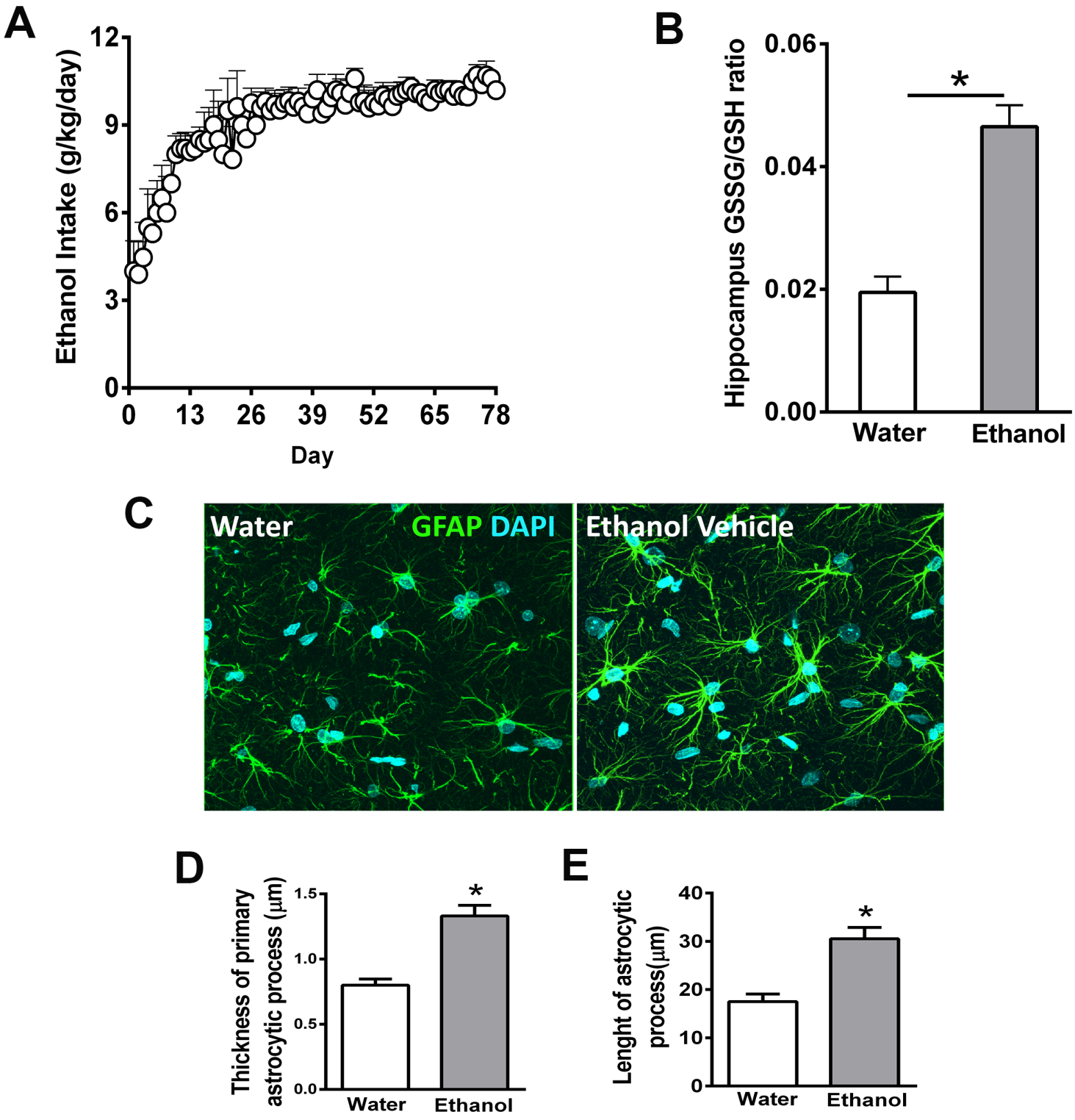

Control-Lenti
Anticatalase-Lenti
Ethanol Intake (g/kg/day)
10
8
6
4
2
-4
4
15
26
37
48
59
DAY
Intra VTA
lentivirus
administration

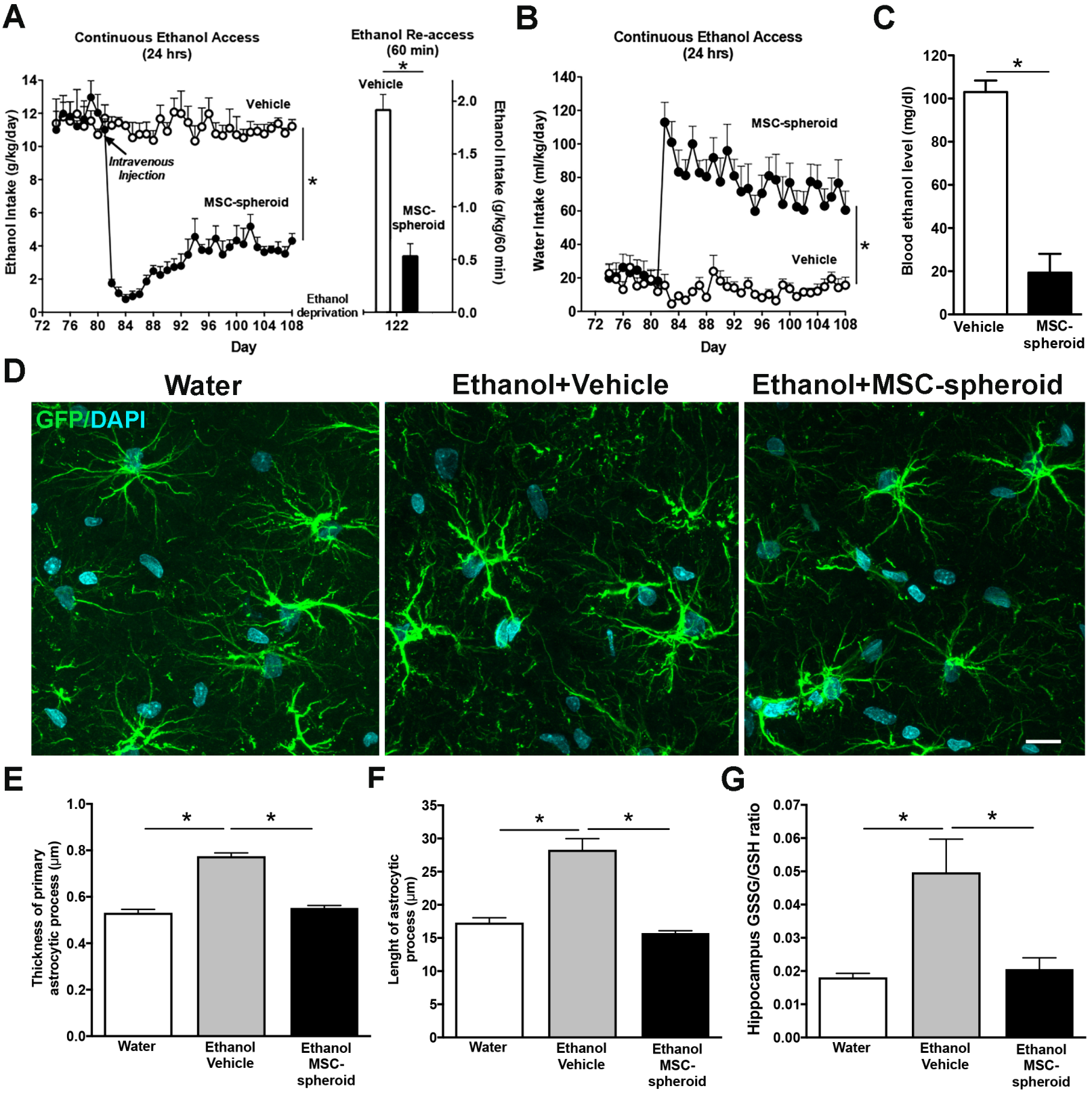

Control-Lenti
ALDH2-Lenti
Ethanol Intake (g/kg/day)
10
8
6
4
2
-4
8
16
24
32
40
48
DAY
Intra VTA
lentivirus
administration

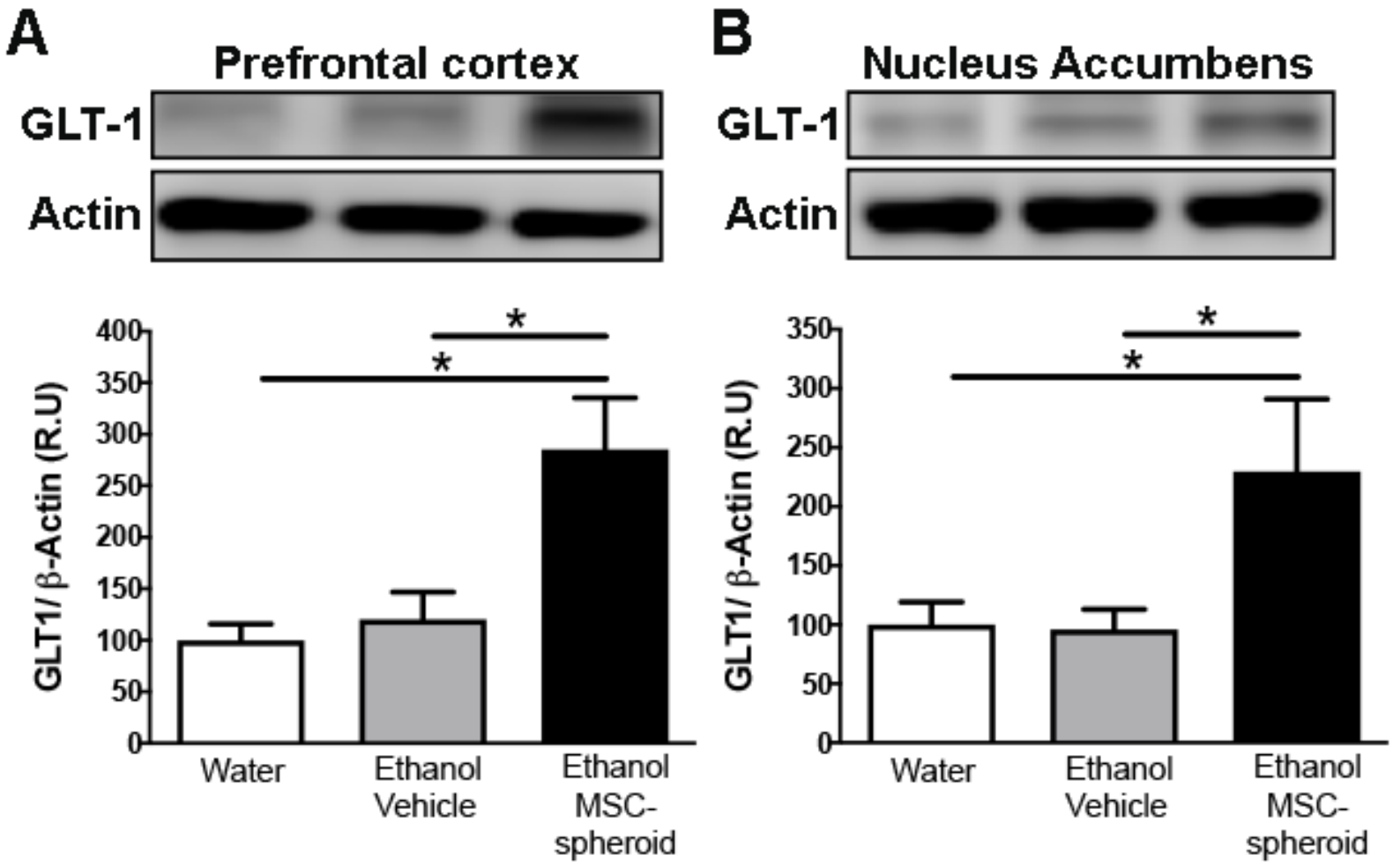

ADH-Lenti
Control-Lenti
Ethanol Intake (g/kg/day)
6
4
2
-4
5
10
15
20
25
30
DAY
Intra VTA
lentivirus
administration

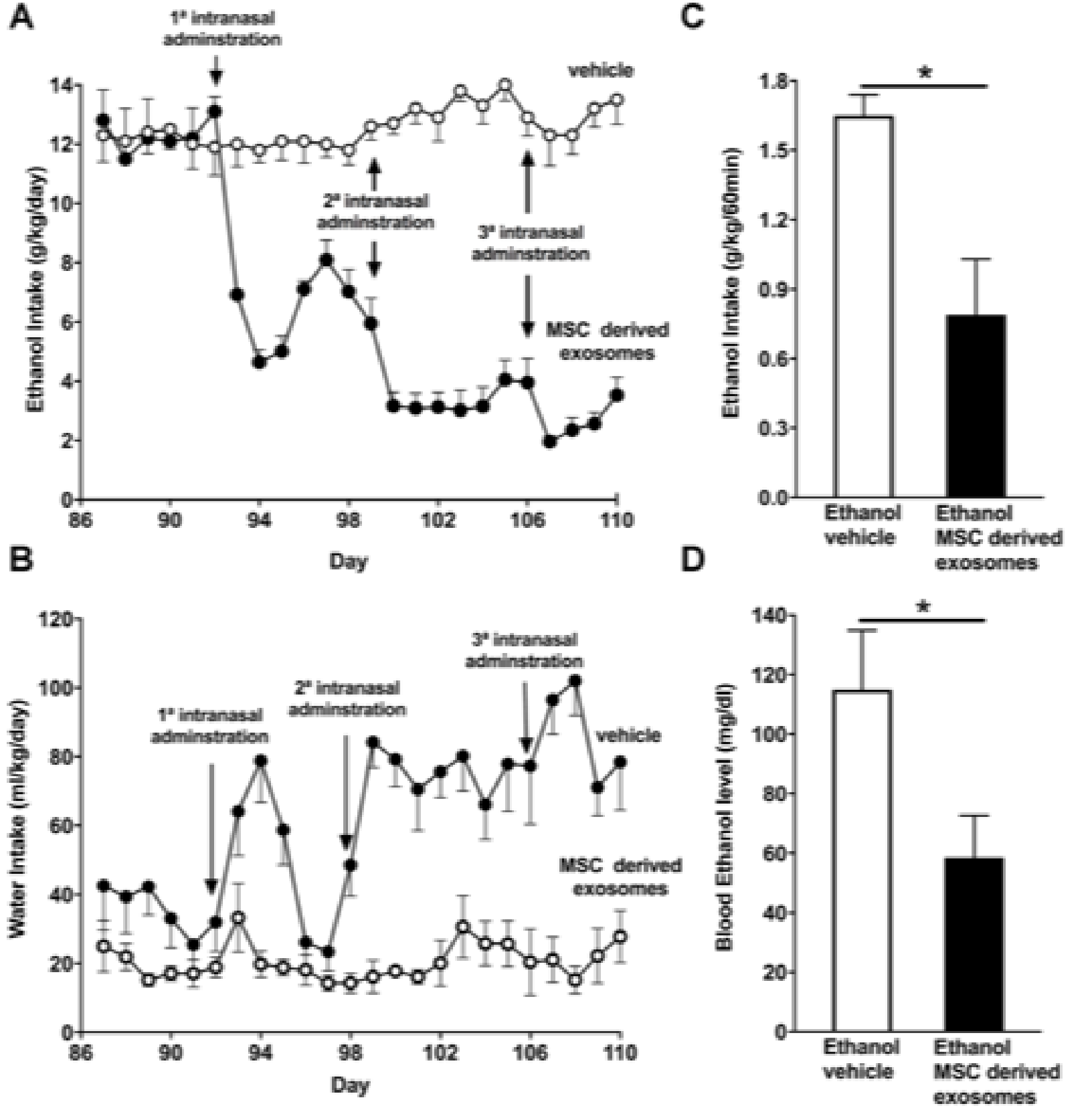

A
Ethanol Intake (g/kg/day)
9
6
3
0
1
13
25
37
49
61
73
Day
Control-Lenti
Anticatalase-Lenti
Intra VTA
lentivirus
administration
B
Ethanol Intake (g/kg/day)
14
12
10
8
6
4
2
0
1
14
27
40
53
66
79
92
Day
Control-Lenti
ALDH-Lenti
Intra VTA
lentivirus
administration


1.8
1.2
0.6
Ethanol Intake (g/kg/60 min)
AdV-control
AdV-ALDH2-AS
Depri-
vation
61
64
67
70
73
76
79
82
85
88
91
94
Day
Intravenous
adenovirous
administration


A
Ethanol Intake (g/kg/day)
12
9
6
3
0
0
13
26
39
52
65
78
Day
B
Hippocampus GSSG/GSH ratio
0.06
0.04
0.02
0.00
*
Water
Ethanol
C
Water
GFAP DAPI
Ethanol Vehicle
D
Thickness of primary astrocytic process (µm)
1.5
1.0
0.5
0.0
*
Water
Ethanol
E
Lenght of astrocytic process(µm)
40
30
20
10
0
*
Water
Ethanol


A
Continuous Ethanol Access
(24 hrs)
Ethanol Intake (g/kg/day)
Vehicle
Intravenous Injection
MSC-spheroid
*
Ethanol deprivation
Day
Ethanol Re-access
(60 min)
Vehicle
MSC-spheroid
Ethanol Intake (g/kg/60 min)
122
B
Continuous Ethanol Access
(24 hrs)
Water Intake (ml/kg/day)
MSC-spheroid
Vehicle
Day
C
Blood ethanol level (mg/dl)
Vehicle
MSC-spheroid
D
Water
Ethanol+Vehicle
Ethanol+MSC-spheroid
GFP/DAPI
E
Thickness of primary astrocytic process (µm)
Water
Ethanol Vehicle
Ethanol MSC-spheroid
F
Lenght of astrocytic process (µm)
G
Hippocampus GSSG/GSH ratio


A
Prefrontal cortex
GLT-1
Actin
GLT1/ β-Actin (R.U)
400
350
300
250
200
150
100
50
0
*
*
Water
Ethanol Vehicle
Ethanol MSC-spheroid
B
Nucleus Accumbens
GLT-1
Actin
GLT1/ β-Actin (R.U)
350
300
250
200
150
100
50
0
*
*
Water
Ethanol Vehicle
Ethanol MSC-spheroid


A
1ª intranasal adminstration
2ª intranasal adminstration
3ª intranasal adminstration
vehicle
MSC derived exosomes
Ethanol Intake (g/kg/day)
Day
B
1ª intranasal adminstration
2ª intranasal adminstration
3ª intranasal adminstration
vehicle
MSC derived exosomes
Water Intake (ml/kg/day)
Day
C
Ethanol Intake (g/kg/60min)
Ethanol vehicle
Ethanol MSC derived exosomes
D
Blood Ethanol level (mg/dl)
Ethanol vehicle
Ethanol MSC derived exosomes