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Reserpine-induced depression is associated in female, but not in male, adolescent rats with heightened, fluoxetine-sensitive, ethanol consumption

Running Head: Sex-related effects of reserpine on alcohol intake

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Research Highlights

- Reserpine and fluoxetine treatment were given to adolescent Wistar rats.
- Reserpine reduced brain dopamine and distance travelled in an open field.
- Reserpine heightened ethanol intake in female, but not in male, adolescents.
- Reserpine heightened risk-taking behaviors in females, but not in males.
- Fluoxetine inhibited the effects of reserpine on ethanol intake and risk-taking.

Abstract

Depression usually emerges during adolescence, is significantly more frequent in women, and exhibits comorbidity with alcohol (ethanol) use disorders. Most of the pre-

clinical studies assessing the link between depression and ethanol intake, however, have employed only males or relied on stress-induced depression, or induced the experimentally-induced, depressive-like phenotype, during adolescence yet measured ethanol intake at adulthood. This study assessed, in Wistar male and female adolescent rats, the effects of inducing experimental depression (via administration of 1.0 mg/kg reserpine [RES], a monoamine depleting drug, between postnatal day [PD] 30 to PD33) on the acquisition of voluntary ethanol drinking during PD38 to PD42), and the modulation of these effects by fluoxetine (FLUOX, 10.0 mg/kg) on PDs 34 to 37. RES-treated rats exhibited a significant reduction of dopamine levels at the insula, no significant changes in circulating levels of thyroxine T4, and reduced distance travelled in an open field. Repeated treatment with RES heightened ethanol intake in female, but not in male, rats; and effect that was inhibited by FLUOX. Similarly, RES significantly increased, and FLUOX reversed, risk-taking behaviors in a concentric square field (CSF) test. FLUOX significantly increased shelter-seeking in the CSF and reduced insular dopamine levels. These results indicate that depression, in females, can kindle the initiation of voluntary ethanol drinking in adolescence (one of the most reliable predictors of being diagnosed with an AUD), and pinpoint alterations in risk-taking as potential mechanisms underlying this effect. Adolescent women afflicted by mood disorders should be specifically targeted for interventions directed towards delaying initiation of alcohol consumption.

Keywords: ethanol; reserpine; fluoxetine, adolescents; experimental depression

1. Introduction

In most western countries alcohol (hereinafter referred to as ethanol in the context of preclinical studies) intake begins at age 13-14 and is almost normative by the end of high school [1, 2]. Despite this, only a subset of adolescents will exhibit alcohol

use disorders (AUD) at adulthood. It is thus important to identify factors that discriminate between those adolescents that will exhibit problematic alcohol use from those that, despite similar initial exposure to the drug, will keep non-problematic alcohol patterns.

Mood disorders (mania and depression) exhibit high comorbidity with AUD [3]. The reasons underlying the association between mood disorders, notably depression, and AUD are not well understood, although a long-standing theory [the "self-medication hypothesis" [4, 5] suggests that alcohol intake in depressed individuals is driven by the pharmacological effects of the drug, as a mean to restore normal mood functioning. The interest in these ideas has been reenacted by a more recent theory ("drug instrumentalization theory"), which proposes that drugs are used for their effects on mental states [6, 7]. A recent study has provided some plausible mechanisms for these proposals. Specifically, ethanol drinking restored the homeostasis of sphingolipids in the brain membranes of mice exhibiting an anxiety-like phenotype [8]

Several pre-clinical studies have reported facilitatory effects of experimental depression on seeking and intake of ethanol or other drugs. For instance, olfactory bulbectomized rats show several biochemical and behavioral alterations that parallel those of human depression, and also exhibit greater self-administration of amphetamine [9], metamphetamine [10], cannabinoid receptor agonists [11] and ketamine [12], than control counterparts.

Some of the researchers that assessed the link between ethanol intake and experimental depression employed strain of rats that, innately or after selective breeding, show a depressive-like phenotype [13, 14]. Illustrating this point, a study selectively bred rats for high or low performance in an escape task [15]. The offspring of breeders with low performance in the task exhibited reduced sucrose intake, an

indicator of anhedonia, and drank significantly more ethanol than controls. Yet the vast majority of the animal studies assessing the link between experimental depression and ethanol intake induced depression by chronically exposing the rodents to aversive events (i.e., stress sources: social instability, foot-shock, physical restraint or prolonged lack of maternal care). For instance, rats were exposed to daily episodes of maternal separation or to standard home cage conditions during postnatal days (PDs) 2-14 [16]. As adults these animals drank significantly less sucrose yet drank significantly more ethanol than controls, and also exhibited signs of anxiety in the elevated plus maze. These effects were reversed by paroxetine, a serotonin reuptake inhibitor used to treat depression in humans. Other studies found that prolonged isolation yields a depressive-like state expressed through anhedonia, anxiety and aggression [17, 18] and heightened ethanol intake [19-21]. Illustrating this point, Wistar rats were exposed to social defeat and isolation for more than two months [22]. This procedure yielded a depressive-like phenotype that was associated with heightened self-administration of ethanol.

The use of stress exposure to assess the interactions between experimental depression and ethanol intake is, however, problematic. The effects of stress on animal models of ethanol consumption are complex. Stress has been observed to increase, decrease or have no effect upon ethanol consumption, as a function of parameters such as length and type of stressor, or strain of rodents used (for review and references, see [23]). Moreover, it is unknown if results observed after stress-induced experimental depression can be applied to depressive-like states that emerge outside of a prolonged exposure to aversive stimulation.

Mood disorders usually emerge, similar to anxiety but different to impulse control disorders, during adolescence [24], and are much more frequent in women than in men [25], particularly after the onset of puberty [26]. Despite this age- and sex-

effects, the vast majority of pre-clinical studies on the link between experimental depression and ethanol intake have employed males and, to our knowledge, almost none conducted both events (induction of experimental depression and measurement of ethanol intake) within the adolescent stage of development. As described above, some studies have induced experimental depression in childhood [16] or early adolescence [18] and then tested preference for ethanol at adulthood. These designs are valuable to understand the lingering effects of adolescent depression, yet they fell short in terms of modelling the trajectories of alcohol consumption and emergence of mood disorders, found in humans.

To sum up, more work is needed to determine ethanol use liability in adolescents exhibiting mood disorders, and sex differences in this phenomenon should be carefully scrutinized. Males and females exhibit several differences in reactivity to ethanol. We recently reported, for instance, that adolescent females significantly increased ethanol intake after exposure to restraint stress, yet the same stressor significantly reduced ethanol intake in adolescent males [27], whereas in other study we found greater ethanol intake after repeated treatment with amphetamine in adolescent males, but in adolescent female, rats [28]. Despite this and other studies, and the explicit indication of funding agencies to include equal number of male and females in epidemiological and pre-clinical research designs [29], women/female are still underrepresented.

The relevance of assessing ethanol use, and sex related differences, in adolescents afflicted by psychiatric disorders is highlighted by two recent studies. One of these [30] found similar ethanol intake in rats with neonatal ventral hippocampal lesions (NHVL), a model of schizophrenia, and in control rats. NHVL rats, however, exhibited greater ethanol intake than controls after a brief exposure to ethanol during

adolescence. The other study [31] tested ethanol intake in adult rats derived from a pharmacological model of schizophrenia. The study revealed no sex-differences in basal ethanol drinking, but heightened ethanol drinking, in females only, after a period of forced abstinence.

Experimental depression in rodents can also be induced by administration of pharmacological agents, such as reserpine (RES [32, 33]), a drug that depletes brain monoamines by disrupting vesicular storage of these transmitters. For instance, mice given reserpine (2.0 mg/kg/day) exhibited behaviors indicative of anxiety (reduced time spent in the open arms of an elevated plus maze) and a depressive-like phenotype (increased immobility in the tail suspension and forced swimming tasks) [34]. In a previous study [35] we found that adolescent male rats treated with RES (0.0 or 1.0 mg/kg/day, postnatal days 30 to 33) exhibited, when compared to controls, reduced motor activity, alterations in the sucrose-preference test, reduced insular levels of dopamine and heightened circulating levels of tyrosine T4. Free-choice ethanol intake (concentration: 3%) was tested a few days later. The vehicle-treated, but not the reserpine-administered, rats exhibited a significant decline in ethanol acceptance across days, and this pattern was not affected by treatment with fluoxetine (FLUOX), an antidepressant of the selective serotonin reuptake inhibitor class [36]. This study from our group [35] suggests that the induction of experimental depression at adolescence can alter responsiveness to ethanol, yet it has important caveats. The study employed only males, tested ethanol intake only in three sessions, and used a very low concentration of ethanol.

The main aim of the present study was to assess, in Wistar male and female adolescent rats, the effects of inducing a depressive-like phenotype (via treatment with RES between PD30 to PD33) on the acquisition of voluntary ethanol drinking during

PD38 to PD42, and the modulation of these effects by FLUOX on PDs 34 to 37. It is always difficult to ascertain the extension of adolescence in the rat, yet PDs 28-42 have been proposed to match early/mid-adolescence in humans, the previous week as juvenile period, and the PD 46-59 period as late adolescence [37-40]. Under this framework, all treatments and analysis of the present study were conducted during early/mid-adolescence. We also measured, after RES and FLUOX treatment, insular levels of dopamine (DA), circulating levels of thyroxine 4 (T4) and risk-taking, shelter-seeking and exploratory behaviors in a concentric square field (CSF) apparatus.

DA transmission occurs in several brain areas and plays a key role in the regulation of mood disorders and ethanol preference. We decided to focus on the insular cortex, however, based on our observation of altered ethanol consumption and low level of dopamine at the insula in adolescent rats [35]. Moreover, it has been shown that alterations in the structure or function of the insular cortex have a significant role in the neurobiology of depression [41]. For instance, patients with major depression exhibit, when compared with healthy controls, a reduction of the gray matter at the insular cortex [42]. A recent pre-clinical study has also indicated that, in the chronic mild stress model of depression, the connectivity between the raphe nucleus and the insular cortex was reduced [43]. Also, in our previous study we found [35] altered levels of T4 after the RES treatment and others have reported both hyper [44] and hypothyroidism [45] in individuals afflicted with depression. Specifically, several studies have reported alterations, mainly increments, in the functioning of the hypothalamic–pituitary–thyroid axis, in patients afflicted by different psychiatric conditions, notably depression. Moreover, it has been suggested that the level of T4 may serve as a predictor of response to antidepressive treatments [46]. Given this background, we considered important to measure the levels of T4 in our animal model of experimental depression.

2. Material and Methods

2.1 Overall description of experiments, experimental designs and subjects

In Experiment 1 the rats received daily administration of RES or vehicle, during four days, and then were tested for ethanol intake across five free-choice intake tests (Exp. 1a) or, to assess the effectiveness of RES to induce a depressive-like phenotype, for motor activity in an open field (Exp. 1b). After finding that RES heightened ethanol intake and preference in female but not in male rats, Experiment 2 analyzed insular levels of DA and circulating levels of T4 after RES and FLUOX treatment. In Experiment 3 we assessed if the rats treated with RES/FLUOX would exhibit alterations in risk-taking, shelter-seeking behavior and, more broadly, in exploratory behaviors after placement in a challenging new environment. We employed a CSF apparatus, which combines the layout of the light-dark box test and the open field test, in conjunction with the possibility of exploring a sector which is only accessible via jumping and an elevated and brightly illuminated sector [47].

The experiments employed a 2 (sex: male or female) x 2 [reserpine treatment at PDs 30-33: reserpine or vehicle, RES or VEH] x 2 [fluoxetine treatment at PDs 34-37: fluoxetine or vehicle, FLUOX or VEH] factorial design. Experiments 1a and 3 had 9-10 and 6-8 subjects in each group, respectively. The 8 groups of Experiment 1b and Experiment 2 had 7-8 and 8-10 subjects, respectively. All rats were experimentally naïve at the beginning of the experiments (i.e., there was no reuse of animals from one experiment to the other).

The animals were Wistar male and female rats born and reared at one of the vivariums of INIMEC-CONICET-National University of Córdoba (UNC, Córdoba,

Argentina). The vivarium is kept at 22 ± 1ºC, on a 12 hr. light/dark cycle (0800). Births were inspected daily between 900 AM and 200 PM and the parturition day was considered PD0. Weaning was conducted day at PD21, when animals were transferred in same-sex groups of four to standard cages (45 x 30 x 20 cm) with ad libitum food and water. Experimental procedures began at PD30. Litter effects were minimized by assigning only one subject per litter to a given experimental condition [48]. All procedures complied with the Guide for Care and Use of Laboratory Animals [49], as adopted and promulgated by the NIH and the EU, and were certified by the Institutional Animal Care and Use Committee at INIMEC-CONICET-UNC. The experiments also complied with the Declaration of Helsinki.

2.2 Drug preparation and administration procedures

Across experiments, the animals were given repeated treatment with reserpine (1.0 mg/kg, i.p., volume of 0.01 ml/g; Sigma-Aldrich, USA) or its vehicle, one dose daily on PDs 30 to 33. In Experiments 1a, 2 and 3 this was followed by administration of fluoxetine (10 mg/kg, i.p., volume of 0.05 ml/g; Eurofarma, Uruguay), once a day on PDs 34-37. Distilled water was the vehicle for both drugs. The reserpine dose was chosen based on studies from our lab [35], showing that reserpine induced a depressive-like phenotype in Wistar adolescent rats, as shown in open field, sucrose intake and light-dark box tests. Fluoxetine dose was chosen based on previous preclinical literature [50, 51] indicating its effectiveness to reduce depressive-like phenotypes. Both reserpine and fluoxetine were given at about 900 AM.

2.3 Ethanol Intake tests (Experiment 1)

In Experiment 1a the rats were treated with reserpine or fluoxetine and then tested for ethanol acceptance. The ethanol intake protocol consisted on 5 daily sessions (PDs 38-42, 2h each) of free-choice (ethanol vs. water tube) drinking. This test has been regularly used in our lab and has proven reliable to detect, among others, stress exposure effects upon ethanol intake and preference [52, 53]. Briefly, animals were weighed and individually placed for two hours in wire mesh cages, equipped with two graduated glass intake tubes (volume: 25 ml; graduation: 0.1 ml; one filled with ethanol, the other with vehicle). Testing began each day at 900AM, after animals had undergone overnight liquid deprivation. The ethanol tube contained 3% on PD38, 4% on PDs39-41 and 5% on PD42 (vehicle: tap water). These concentrations are similar to the ethanol content of beverages that are preferred by human adolescents [54, 55] and are readily consumed by heterogeneous [56] Wistar rats obviating the need for sucrose additives to encourage drinking. Also, compared to traditional 24-h intake tests this had the advantage that rats concentrate most of their drinking during the first 20 minutes of the test, thus ensuring that they are experiencing the pharmacological, post-absorptive, effects of ethanol [57]. The readings obtained from each tube (i.e., ml ingested) at the end of each daily session were used to calculate g/kg of ethanol ingested and % preference for the ethanol solution. Measurement error due to leakage was taken care of by placing two graded tubes (one containing vehicle, one containing ethanol) in an empty cage. The readings obtained from these tubes were subtracted from those registered in the cages with animals.

2.4 Measurement of distance travelled in an open field (Experiment 1b)

In Experiment 1b a group of male and female rats, separate from those that underwent ethanol intake testing in Exp. 1a, were given reserpine on PDs 30 to 33 and,

on PD 34, were assessed for distance travelled in an open field maze, fully described in [58]. Briefly, the animals were withdrawn from their cages and placed in opaque (60 cm× 60 cm × 60 cm) open field (OF) chambers made of Plexiglas and equipped with photocell beams. Beam breaks were recorded during five minutes by an activity monitoring system (ITCOMM, Córdoba, Argentina) which yielded a measure of horizontal distance traveled (cm).

2.5 Measurement of dopamine and thyroxin (T4) levels (Experiment 2)

In Experiment 2, dopamine levels at the insula and trunk blood levels of tyrosine were measured from samples collected at postnatal day 38, 24h after the last administration of fluoxetine. We followed procedures similar to those described in [35]. The rats were killed by decapitation. Immediately, the insular cortex was quickly dissected using the technique described in [59] and trunk blood samples (1.5 ml) were obtained using heparinized tubes.

The insular cortices were stored at -80 C and then were weighed and sonicated in 1ml of perchloric acid (0.1M). A supernatant was obtained by high-speed centrifugation (15000 RMP, 15 min) and then injected into an HPLC system (PM-80 BAS, West Lafayette, IN, USA; settings described in [28]), which provided a measure of dopamine levels (ng/g). The serum, obtained after centrifugation of the blood at 2000 rmp for 20 min, was incubated with radiolabeled hormones and subsequently quantified in a gamma counter. Radio Immuno Assay kits (RIA, Siemens) were employed and free T4 thyroxine levels are expressed as ng/dl [60].

2.6 Test of risk-taking and shelter-seeking behavior.

The rats of Experiment 3 were given reserpine and fluoxetine treatment and, 72h after termination of the pharmacological treatment, were tested, in a single 20-min

session, for risk-taking and shelter-seeking behavior on PD40. The CSF [48 cm × 48 cm × 48 cm, fully described in [27]] maze was a square, wooden box, with a central open field (OF) that connected three corridors. Two of these corridors led to the challenge (CHA) sector, which was accessed only by performing a risk taking behavior (i.e., jump through a hole in the doorway, raised 10 cm above the floor). The other corridor headed to a sheltered (SHEL) sector, which was fully enclosed and was not illuminated. One of the corridors leading to the CHA had a bifurcation that led to a brightly lit section, separated from the exterior by a transparent plastic and equipped with a ramp (R, 12 cm, inclination 20 graded) that headed to an elevated structure (i.e., the BRIDGE). R and BRDIGE were made of metallic mesh and could be climbed and explored. The illumination of each sector was the following: SHEL (0 lux), corridors and CHA sector (20-30 lux), and R and BRIDGE (600-650 lux).

The CSF test lasted 20 min and was videotaped for later assessment of time spent and frequency of entries in the OF, SHEL sector, CHA sector, R, BRIDGE, and in the connecting corridors. More in detail, the session was recorded via a camera fixed in a metal rail hanging from the ceiling. Video files were stored and later analyzed by three trained undergraduate students. These individuals were not aware of the treatment assigned to each of the rats tested.

The time spent and frequency of entries in the SHEL, OF and CHA sections were considered measures of shelter seeking, exploratory behavior and risk taking, respectively. The time spent and frequency of entries in the R and BRIDGE reflected risk taking. Preliminary analysis of the latter variables reflected that both yielded similar profiles. Thus, and for the sake of parsimony, those scores were added up and presented as a single variable (R+BRIDGE). The total number of section entries and the total number of entries into the connecting corridors (measures of overall motor activity)

were also calculated. The CSF has several advantages over traditional tests for the assessment of anxiety, shelter-seeking and risk taking. Among others, the test provides –unlike the light-dark or elevated plus maze tests -- non-binary behavioral options, and the simultaneous nature of the testing (i.e., all behaviors are measured at once) prevents the confounding effect of sequential testing in several apparatuses [61].

2.7 Data analysis

Separate four way-RM ANOVAs (between factors: Sex, Reserpine treatment and Fluoxetine treatment; within-variable: Postnatal day of assessment, PD38, 39, 40, 41 and 42) were used to analyse body weights (g) registered prior to each intake session, as well as absolute (g/kg) and percent (%) ethanol intake scores, and water intake, expressed as ml/100 g of body weight. Independent three-way ANOVAs (between factors: Sex, Reserpine treatment and Fluoxetine treatment) analysed dopamine concentration (ng/g) at insular cortex and free T4 thyroxine (ng/dl), registered in Experiment 2. Similar ANOVAs were used to independently assess each behavior measure in the CSF. Distance travelled in the open field was analysed via a two-way ANOVA, with Sex and Reserpine treatment serving as the between factors.

The loci of significant main effects or significant interactions were analysed via LSD post hoc comparisons, and Cohen's partial eta squared (η^2p) was used to describe effect sizes. Please note that, given the complexity of depicting significant main effects that span several groups and conditions, some of the significant main effects have not been depicted in the figures via asterisks or other signs. Instead, a brief description of these significant differences can be found in each figure legend. An alpha level of 0.05 was enforced for all the analyses.

3. Results

3.1 Experiments 1a and 1b

Ethanol intake on a gram per kilogram basis was greater in female, but in not male, rats treated with reserpine than in their corresponding controls. This sex-dependent effect was inhibited by fluoxetine. These impressions were corroborated by the ANOVA, which yielded a significant effect of Sex and a significant Reserpine treatment x Fluoxetine treatment x Sex interaction [$F_{1,64} = 4.18$, $p < 0.5$, $\eta^2p = 0.06$ and $F_{1,64} = 5.93$, $p < 0.5$, $\eta^2p = 0.08$]. The post-hoc tests indicated that females given RES-VEH drank more than VEH-VEH females and that RES-FLUOX females. Ethanol intake (g/kg) was similar among male groups, regardless RES or FLUOX treatment.

The ANOVA for ethanol percent preference yielded significant main effects of Sex, Reserpine and Session [$F_{1,64} = 4.18$, $p < 0.5$, $\eta^2p = 0.06$ and $F_{1,64} = 5.93$, $p < 0.5$, $\eta^2p = 0.08$]. The interaction between Sex, Reserpine and Fluoxetine was also significant, $F_{1,64} = 5.76$, $p < 0.5$, $\eta^2p = 0.08$. Percent preference for ethanol was, across groups, higher in the first than in the subsequent sessions. Perhaps more important, the *post-hoc* tests indicated that females given reserpine but spared from fluoxetine (i.e., RES-VEH group) preferred more ethanol than VEH-VEH females. The latter, basic control, condition exhibited similar ethanol percent preference as the RES-FLUOX group. RES or FLUOX treatment did not significantly alter ethanol percent preference in males.

Figure 1 depicts the significant three-way (Sex x Reserpine x Fluoxetine) interaction yielded by both ANOVAs and Figure 2 depicts ethanol intake data (g/kg) across testing days in all groups. Percent preference scores are not shown across days.

PLEASE INSERT FIGURE 1 and 2 ABOUT HERE

The ANOVA for body weights indicated significant main effects of Sex, Postnatal day and Reserpine treatment [$F_{1,68} = 32.18$, $p < 0.001$, $\eta^2p = 0.32$; $F_{4,272} =$

39.89, $p < 0.001$, $\eta^2p = 0.37$ and $F_{1,68} = 16.15$, $p < 0.001$, $\eta^2p = 0.19$, respectively]. As expected, females were lighter than males and reserpine induced a significant reduction in body weight, which was fairly similar across days, despite a significant two-way interaction between Postnatal day and Reserpine Treatment, $F_{4,272} = 6.05$, $p < 0.001$, $\eta^2p = 0.08$. The analysis of water liquid intake scores (ml/100 g) yielded significant main effects of Sex, Postnatal day, Reserpine treatment and Fluoxetine treatment, $F_{1,68} = 9.90$, $p < 0.005$, $\eta^2p = 0.13$, $F_{4,272} = 30.68$, $p < 0.001$, $\eta^2p = 0.31$, $F_{1,68} = 45.28$, $p < 0.001$, $\eta^2p = 0.40$ and $F_{1,68} = 14.60$, $p < 0.001$, $\eta^2p = 0.18$, respectively. Water intake was greater in males than females, and in those subjects treated with reserpine or fluoxetine than in vehicle treated counterparts. The reserpine x fluoxetine interaction did not achieve significance (p>.10) nor there were significant interactions between the pharmacological treatments and sex. Body weights and water intake scores are presented on Table 1.

PLEASE INSERT TABLE 1 ABOUT HERE

The administration of reserpine induced a significant reduction in motor activity, as shown by a dramatic reduction in distance travelled (cm) in the open field, measured 24 h after the last injection. The ANOVA revealed a significant main effect of Reserpine treatment, $F_{1,26} = 81.89$, $p < 0.001$, $\eta^2p = 0.76$. No significant main effect of Sex or significant interactions were observed. Mean and SEM in female rats were 2946.88±235.20 and 980.87±159.18, whereas males exhibited 2843.14±299.89 and 848.57±154.50, vehicle- and reserpine-treated rats, respectively

3.1 Experiment 2

As shown in Figure 3A, the administration of reserpine induced a significant drop in dopamine levels (ng/g) at insular cortex. This reduction was also observed,

although to a lesser extent, after administration of fluoxetine only. These impressions were confirmed by the ANOVA, which yielded significant main effects of Reserpine treatment, Fluoxetine treatment and a significant Reserpine x Fluoxetine interaction [$F_{1,50}$ = 34.43, p < 0.001, η^2p = 0.41; $F_{1,50}$ = 7.74, p < 0.01, η^2p = 0.13 and $F_{1,50}$ = 21.13, p < 0.001, η^2p = 0.30, respectively]. The post-hoc indicated significantly reduced DA levels in groups RES-VEH, VEH-FLUOX or RES-FLUOX, when compared to the basal, VEH-VEH, group. DA levels were also greater in the VEH-FLUOX group than in the RES-VEH group. The latter groups, in turn, exhibited similar DA levels as those found in the RES-FLUOX condition. This pattern of results was statistically similar in male and female rats (Figure 3 B-C).

The ANOVA for free T4 thyroxine (ng/dl, see Fig. 4 A-C) scores revealed that, in male and female rats alike, fluoxetine significantly reduced the levels of this hormone [significant main effect of Fluoxetine: $F_{1,37}$ = 4.32, p < 0.05, η^2p = 0.10].

PLEASE INSERT FIGURES 3 AND 4 ABOUT HERE

3.1 Experiment 3

Experiment 3 assessed exploration, shelter-seeking and risk taking behaviors in male and female rats given repeated treatment with reserpine or vehicle, which was then followed or not by fluoxetine. The ANOVAs for total number of section entries (a measure of overall motor activity) indicated a significant main effect of Sex [$F_{1,50}$= 8.89, p < 0.01, η^2p = 0.15] as well as significant Reserpine x Fluoxetine [$F_{1,50}$= 4.53, p < 0.05, η^2p = 0.08], and Reserpine x Fluoxetine x Sex interactions [$F_{1,50}$= 5.51, p < 0.05, η^2p = 0.10]. Mean and SEM number of section entries was as follows: VEH–VEH 111.00±13.08 and 112.50±13.23, RES–VEH 148.00±9.17 and 107.71±9.76, VEH–FLUOX 142.70±6.42 and 112.50±13.23, and RES–FLUOX 114.00±13.14 and 103.17±10.11, in female and male rats, respectively. The *post-hoc* tests revealed that

overall number of entries were statistically similar in male rats regardless treatment with either drug. On the contrary, females treated with 1.0 mg/kg reserpine and 0.0 mg/kg fluoxetine exhibited significantly greater overall activity than females given vehicle-vehicle treatment. Administration of fluoxetine reversed this effect: among female rats treated with 10.0 fluoxetine, reserpine treated rats exhibited significantly less number of entries than peers given 0.0 mg/kg reserpine. A similar pattern (descriptive data not shown) was found when assessing total number of entries into the connecting corridors: significant main effect of Sex ($F_{1,50}$= 5.44, $p < 0.05$, $\eta^2p = 0.10$) and significant Reserpine x Fluoxetine x Sex interactions ($F_{1,50}$= 5.99, $p < 0.05$, $\eta^2p = 0.10$).

The corresponding ANOVAs indicated that females exhibited significantly more time spent or frequency of entries in the SHEL [significant main effects of Sex ($F_{1,50}$= 48.60, $p < 0.001$, $\eta^2p = 0.43$ and $F_{1,50}$= 45.46, $p < 0.001$, $\eta^2p = 0.48$, respectively)] and in the CHA sector [significant main effects of Sex ($F_{1,50}$= 5.21, $p < 0.05$, $\eta^2p = 0.09$ and $F_{1,50}$= 5.85, $p < 0.05$, $\eta^2p = 0.10$, respectively; see Figure 5), than males; and that treatment with fluoxetine increased the time spent in the SHEL [significant main effect of Fluoxetine ($F_{1,50}$= 14.39, $p < 0.001$, $\eta^2p = 0.22$)]. The ANOVAs revealed that males also spent more time in the starting, OF area than females ($F_{1,50}$= 4.33, $p < 0.05$, η^2p = 0.08; Figure 5C-D), whereas the analysis of frequencies of entries in this area did not yield significant main effects or significant interactions (Figure 5A-B).

The ANOVA for frequency of entries in the R+BRIDGE, risk taking, section revealed a significant main effect of Sex [$F_{1,50}$= 13.31, $p < 0.001$, $\eta^2p = 0.21$] as well as significant Reserpine x Fluoxetine [$F_{1,50}$= 9.26, $p < 0.005$, $\eta^2p = 0.16$], and Reserpine x Fluoxetine x Sex interactions [$F_{1,50}$= 4.75, $p < 0.05$, $\eta^2p = 0.09$]. The *post hoc* tests indicated that, similar to what was found in in terms of overall motor activity, the frequency of entries in males was not significantly affected by reserpine or fluoxetine

treatment. In sharp contrast, treatment with 1.0 mg/kg reserpine and 0.0 mg/kg fluoxetine (i.e. RES-VEH group) to the females significantly heightened the number of entries into the R+BRIDGE section, when compared to same-sex peers given vehicle-vehicle treatment. Fluoxetine reversed this effect: Vehicle-fluoxetine treated rats exhibited significantly more number of entries than peers given reserpine and fluoxetine. These results are depicted in Figure 5A-B. The ANOVA for time spent in the R+BRIDGE yielded a significant main effect of Sex ($F_{1,50}$= 7.13, $p < 0.05$, η^2p = 0.13) and a trend towards a significant main effect of Reserpine ($F_{1,50}$= 3.04, p = 0.08, η^2p = 0.06). Time spent in the R+BRIDGE section (see Figures 5C-D) was greater in females than in males and in rats, males or females, treated with reserpine.

PLEASE INSERT FIGURE 5 ABOUT HERE

4. Discussion

The main new finding of the present study is that the induction of a depressive-like phenotype at adolescence enhanced ethanol intake during late adolescence, in female Wistar rats. The effect of RES on the average level of ethanol intake of the females was substantial: they exhibited a two-fold increase in ethanol intake and acceptance, when contrasted with controls unexposed to RES. The effectiveness of RES to induce experimental depression was corroborated in the open field, and neural and hormonal differences between RES and VEH groups were observed. The exploratory behaviors measured in the CSF also shed light on the potential mechanisms underlying the greater ethanol intake in RES-treated females. We will now describe these main findings, and their implications.

The promoting effect of RES on ethanol intake was significantly inhibited by FLUOX and was not found among males, which also drank significantly lower

quantities of ethanol than did females. The latter results are consistent with several pre-clinical studies showing that, in a variety of test situations and unlike the pattern found in humans [1], female rats drank more than male counterparts [31, 62, 63], and that selective serotonin reuptake inhibitors are useful to block ethanol intake [64, 65], particularly when the latter is comorbid with a depressive-like phenotype [16]. Moreover, the insensitivity of males to the promoting effect of RES is relatively consistent with a previous study from our lab [35], in which we found very small and subtle difference between RES (1.0 mg/kg) and vehicle-treated male Wistars.

Overall, these results are important because they suggest that adolescent depression, in females, can kindle the acquisition of voluntary ethanol drinking. An early onset of alcohol drinking is, in turn, one of the most reliable predictors of being diagnosed with an AUD [1, 66]. Thus, these results suggest that adolescent women afflicted by mood disorders are at heightened risk for initiation or escalation of alcohol intake and should be specifically targeted for intervention. It is also important to consider that chronic alcohol intake may further exacerbate a depressive-like phenotype [67].

RES depleted the insular levels of dopamine and FLUOX had a similar lowering action upon dopamine levels, although the effect of FLUOX on DA was significantly lower than that of RES. The effect of FLUOX on DA levels is a puzzling finding and contrast with previous studies indicating that acute FLUOX, at the dose employed in the present study, raised extracellular brain dopamine levels, albeit in the prefrontal cortex [68-70]. Yet others have observed that FLUOX has an inhibitory effect on endogenous or exogenous (i.e., after the administration of L-DOPA) DA levels and can induce or exacerbate parkinsonism [71, 72]. A study that employed a rodent model of Parkinson and depression observed that FLUOX (2.5 – 10 mg/kg) exacerbated DA depletion in the

striatum and exacerbated the motor symptoms [73]. The different outcomes reported, concerning the effect of FLUOX on DA levels, may relate to the site of the measurement. Most of the studies that reported increased DA after FLUOX measured the effect at prefrontal cortex, whereas those reporting reduced DA measured the transmitter at the striatum.

The present study, which measured insular levels of DA, adds further evidence suggesting that FLUOX alters DA levels, and generalizes these findings to the adolescent stage of development. There was, however, dissociation between the effects of FLUOX on ethanol intake, which were observed in females only, and the effects of FLUOX on DA levels, which were fairly similar in males and females. FLUOX also increased the time spent in SHEL, a behavior indicative of heightened anxiety and exerted a subtle, yet significant effect, on the circulating levels of T4. Although it is tempting to link the latter effect to its inhibition of ethanol ingestion in the females, in the present study the rats exhibiting experimental depression did not have, unlike in our previous report [35] alterations in T4, nor the FLUOX-induced change in T4 was sex-dependent. Also relevant is that frequency of entries into the R+BRIDGE was similar in VEH-FLUOX and RES-VEH groups. When this is considered in conjunction with the FLUOX-induced reduction of DA insular levels and the FLUOX-induced enhancement of time spent in the SHEL, it is tempting to argue that FLUOX may have exerted, when not combined with RES, some negative side effects (i.e., greater anxiety or heightened impulsivity) in the adolescent rats of this study. This is, of course, just a hypothesis that should be carefully analyzed in future studies.

What mechanisms led to the increased, RES-induced, ethanol intake found in Experiment 1? One possibility is that RES enhanced anxiety response. Anxiety and depression often co-occur and ethanol exerts anti-anxiety effects at doses similar to

those ingested by the females of Experiment 1. Yet time spent in the enclosed section of the CSF, a measure of shelter-seeking akin to time spent in the closed arms of an elevated plus maze, was not altered by RES. On the contrary, females – but not males -- treated with 1.0 mg/kg RES and 0.0 mg/kg FLUOX exhibited significantly greater overall activity (total number of section entries and total number of entries to corridors) and significantly more frequency of entries into the potentially dangerous areas of R and BRIDGE. Administration of FLUOX to the females reversed these effects of RES, mirroring the pattern of effects found for ethanol intake and preference. In other words, in females only there was an association between RES-induced heightened ethanol intake and heightened exploration of the CSF apparatus in general and, more specifically, of areas of this maze that entail potential risk.

Based on these results, it could be proposed that the depression-like state induced by RES was also accompanied by either deficits in assessment of risk or in greater impulsivity or risk-taking, and that these effects, in turn, drove ethanol intake. This is, of course, just a hypothesis that should be scrutinized in future studies. Yet, it is notable that, in previous pre-clinical studies, we have already found an association between risk taking behavior in the CSF and heightened ethanol intake. In one of these studies rats that had been exposed to environmental enrichment exhibited greater ethanol intake and greater time spent in the CHA sector of the CSF, when compared to peers reared under standard animal rearing conditions[74]. In another study [27] female rats exposed to restraint stress showed significantly greater ethanol intake than non-stressed controls, an effect that was associated with greater time spent in a risk section of the CSF. Impulsivity [47, 75-79] and alterations in risk-taking or assessment are traits associated with propensity for ethanol intake in clinical and pre-clinical studies.

The present results should be considered in the context of several, important,

limitations. Only one dose of each drug (RES and FLUOX) was used, which hinders the scope and applicability of the results. Moreover, RES is a highly promiscuous agent that acts on all monoamines. Future studies assessing the link between mood disorders and ethanol intake in adolescence would benefit from drugs that specifically depletes a given transmitter, such as DL-P-chlorophenylalanine (PCPA), a reversible inhibitor of serotonin synthesis [80]. Moreover, RES also enhanced intake of water and reduced body weight. It could be proposed that the effects of RES upon ethanol intake were unspecific, and resulted from a general increase in seeking of liquids. This is, however, unlikely, as the effects of RES upon body weight and water intake were insensitive to FLUOX and were found in both males and females. Yet, RES-induced facilitation of ethanol intake was exclusively for females and was inhibited by FLUOX. Thus, there seems to be dissociation between the ability of RES to alter body weight and water intake, and its effects upon ethanol consumption. Another limitation is that ethanol intake was tested during the daylight section of light cycle, when animals are relatively less active compared to the dark section of the cycle. Future studies should consider the use of a drinking-in-the dark approach [81] for testing ethanol intake. Last but not least, we did not measure blood ethanol levels among the groups. This as a significant limitation and possible confounding factor, as it is possible that the heightened drinking in RES-treated females was the result of alterations in the metabolism of ethanol.

Despite these limitations, the main contribution of the study is that a short treatment with RES during the adolescence of the rat induces a depressive-like state expressed at the neural and behavioural level. In females, but not in males, the depressive-like state induced by reserpine was associated with heightened ethanol intake during late adolescence, and with a performance in the CSF of increased overall activity

and greater propensity to explore risk areas. FLUOX inhibited the heightened ethanol intake and the alterations in the exploratory patterns.

Declaration of interest: The authors declare no competing interest or conflict of interest related to the data presented in the manuscript.

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FIGURE LEGENDS

Figure 1: (A-D) Ethanol intake (g/kg) (A-B) and percent preference (C-D) in male and female Wistar rats as a function of reserpine (RES) treatment administered on postnatal days 30 to 33 (0.0 [VEH] or 1.0 mg/kg RES] and fluoxetine (FLUOX) treatment administered on postnatal days 34 to 37 (0.0 [VEH] or 10.0 mg/kg FLUOX]. Two-bottle intake sessions (ethanol *vs.* plain water) were conducted each day for five days. The data are expressed as the mean ± SEM across the five sessions. The statistical analysis revealed that male rats give RES and VEH exhibited significantly more ethanol intake (g/kg) than rats in groups VEH-VEH or RES-FLUOX. These significant differences are indicated by the asterisk and pound signs of panel A, respectively. The group RES-VEH also exhibited significantly more percent ethanol intake than rats in group VEH-VEH, as denoted by the asterisk in panel C.

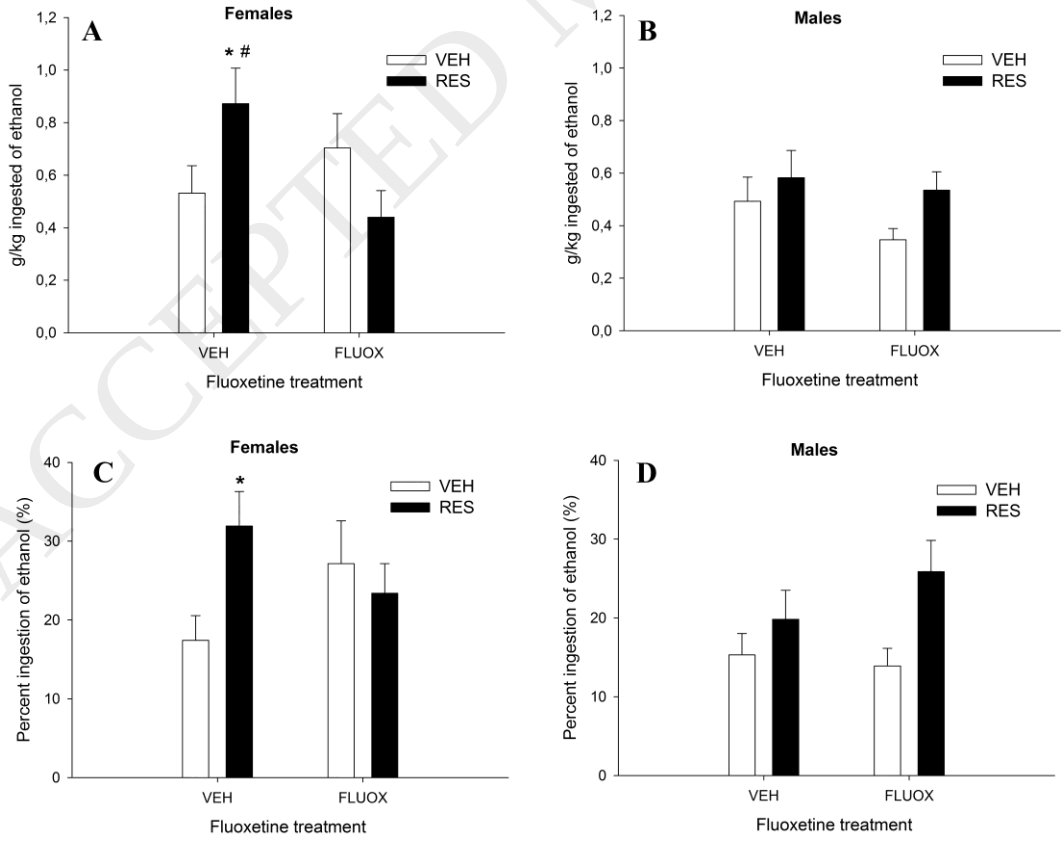

Figure 2: Ethanol intake (g/kg) in male and female Wistar rats as a function of reserpine (RES) treatment administered on postnatal days 30 to 33 (0.0 [VEH] or 1.0 mg/kg RES], fluoxetine (FLUOX) treatment administered on postnatal days 34 to 37 (0.0 [VEH] or 10.0 mg/kg FLUOX], and day of assessment. Two-bottle intake sessions (ethanol *vs*. plain water) were conducted each day for five days. The data are expressed as the mean ± SEM in each of the five sessions.

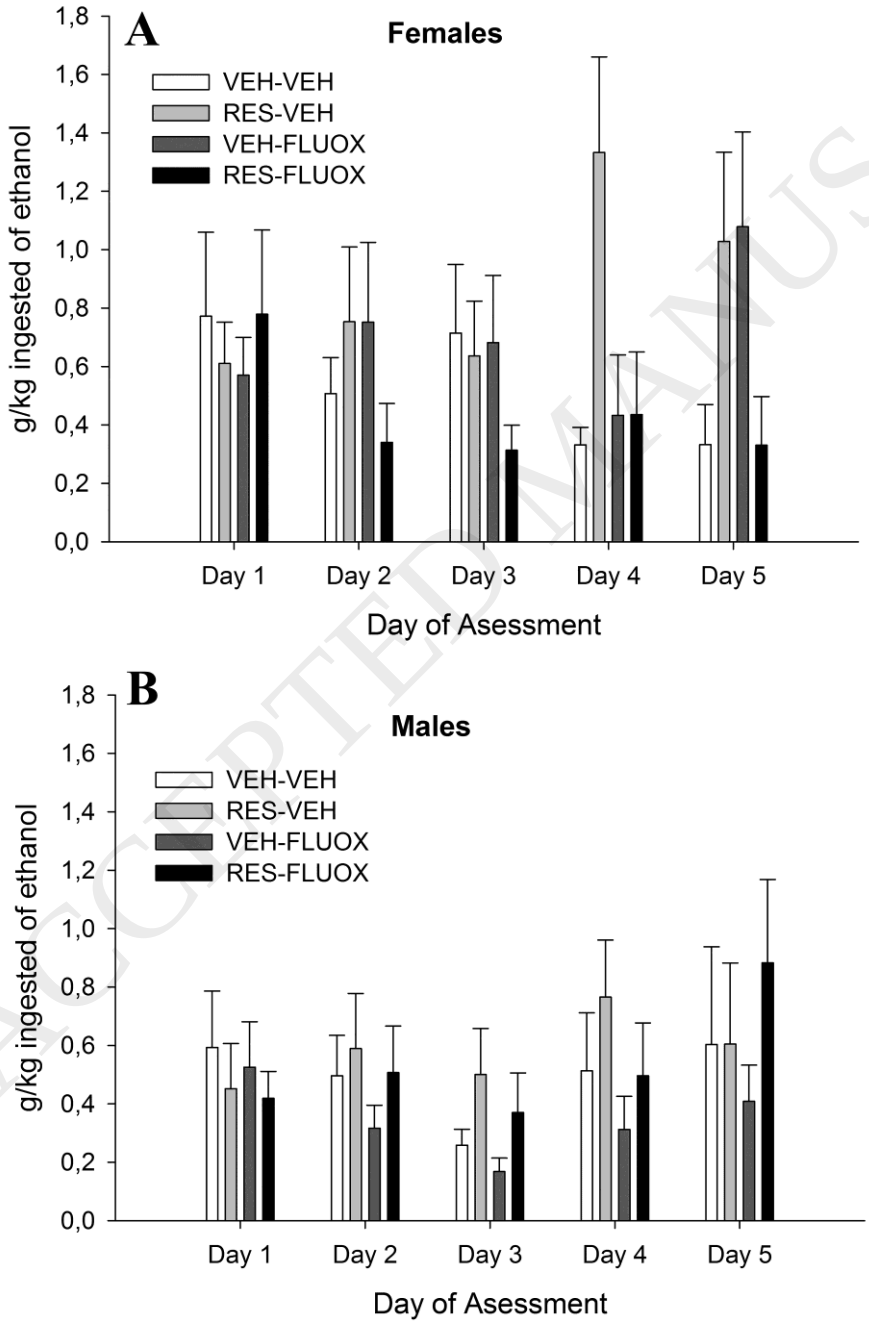

Figure 3: (A) Dopamine levels (ng/g of tissue) as a function of reserpine (RES) treatment administered on postnatal days 30 to 33 (0.0 [VEH] or 1.0 mg/kg RES] and fluoxetine (FLUOX) treatment administered on postnatal days 34 to 37 (0.0 [VEH] or 10.0 mg/kg FLUOX]. The statistical analysis indicated significantly reduced DA levels in groups RES-VEH, VEH-FLUOX or RES-FLUOX, when compared to the basal, VEH-VEH, group. These significant differences are indicated by the asterisk. DA levels were also greater in the VEH-FLUOX group than in the RES-VEH group, as denoted by the pound sign. (B-C) Same information as in A but disaggregated by sex (male, female). The data are expressed as the mean ± SEM.

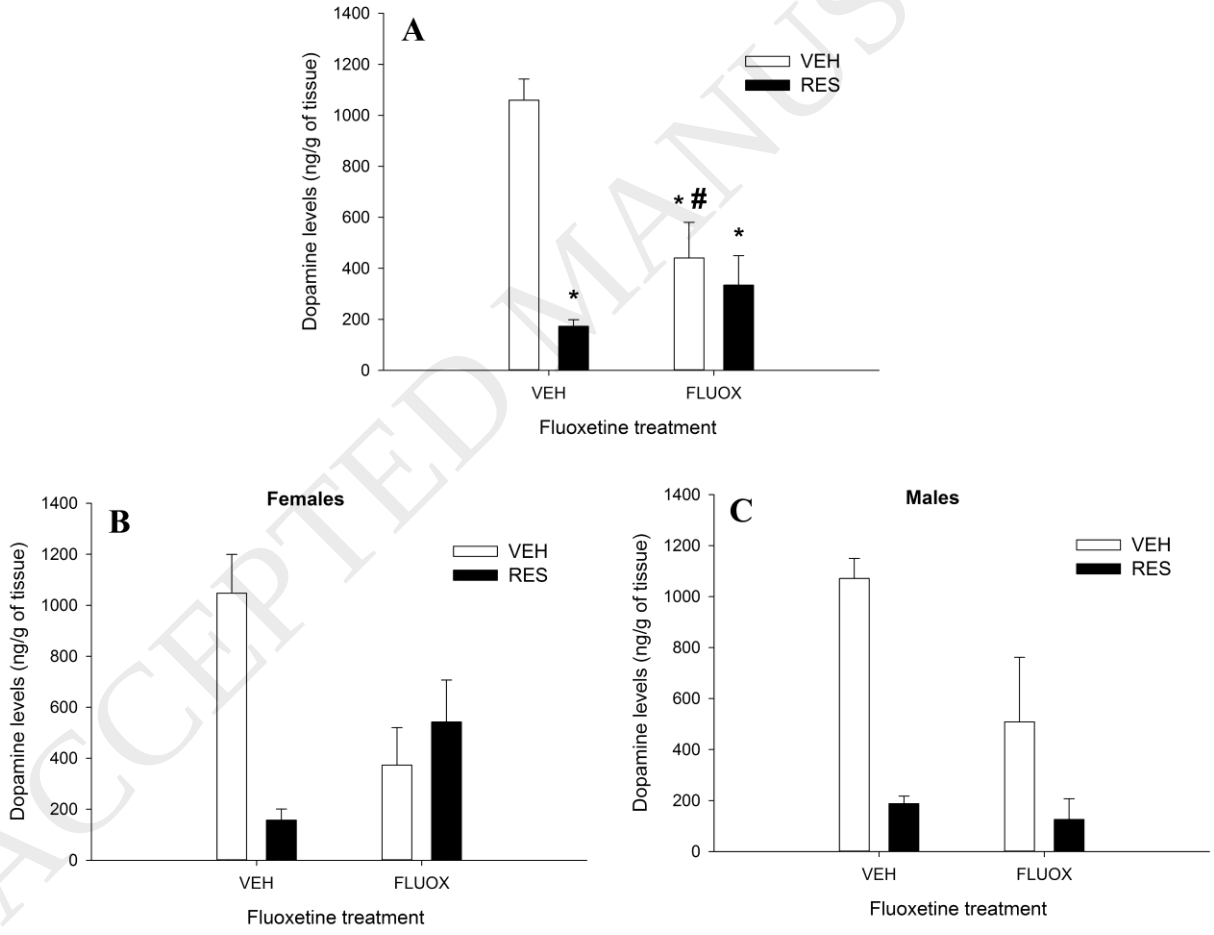

Figure 4: (A) T4 thyroxine levels (ng/g of tissue) as a function of reserpine (RES) treatment administered on postnatal days 30 to 33 (0.0 [VEH] or 1.0 mg/kg RES] and fluoxetine (FLUOX) treatment administered on postnatal days 34 to 37 (0.0 [VEH] or

10.0 mg/kg FLUOX]. The statistical analysis indicated that fluoxetine significantly reduced the levels of T4, as denoted by the asterisk sign. (B-C) Same information as in A but disaggregated by sex (male, female). The data are expressed as the mean ± SEM.

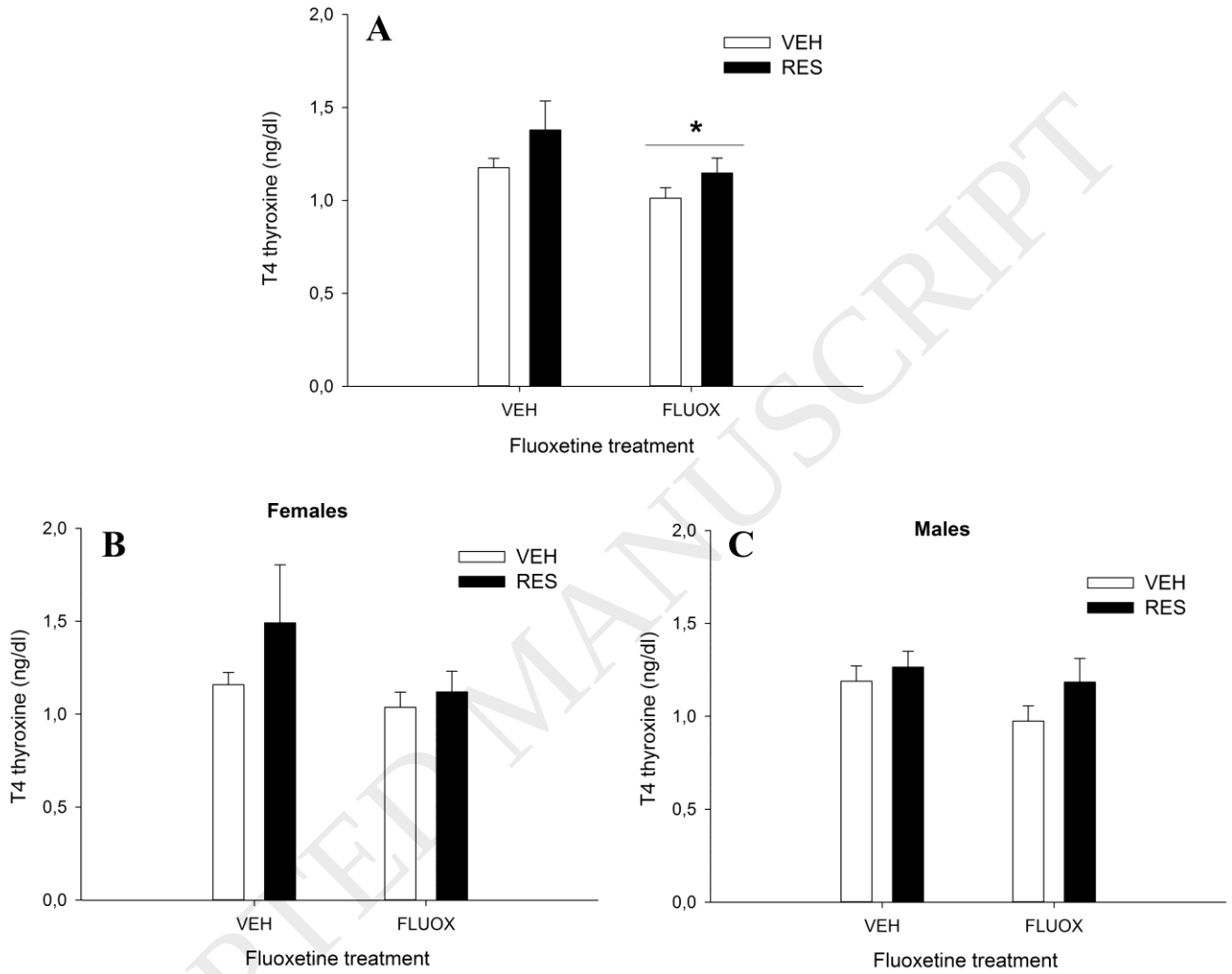

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Figure 5: Frequency of entries (A-B) or time spent (C-D) in the different sections of the concentric square field (CSF) maze [open field (OF), enclosed shelter (SHEL), challenge area (CHA) and ramp and bridge area (R+BRIDGE)] in male and female rats as a function of reserpine (RES) administered on postnatal days 30 to 33 (0.0 [VEH] or 1.0 mg/kg RES] and fluoxetine (FLUOX) treatment administered on postnatal days 34 to 37 (0.0 [VEH] or 10.0 mg/kg FLUOX]. Females treated with 1.0 mg/kg reserpine and 0.0 mg/kg fluoxetine exhibited significantly greater number of section entries than

females given VEH-VEH treatment; and RES-FLUOX female rats exhibited significantly less number of entries than VEH-FLUOX peers. Females exhibited significantly more time spent or frequency of entries in the SHEL and in the CHA sector, than males; and FLUOX increased the time spent in the SHEL. Males also spent more time in the starting, OF area than females. Females in the RES-VEH group exhibited significantly greater number of entries into the R+BRIDGE section than females VEH-VEH, and VEH-FLUOX females exhibited significantly more of this behavior than RES-FLUOX peers. These differences are a difference are indicated by the asterisk and pound sign, respectively. Time spent in the R+BRIDGE section was greater in females than in males and in rats. The data are expressed as the mean ± SEM.

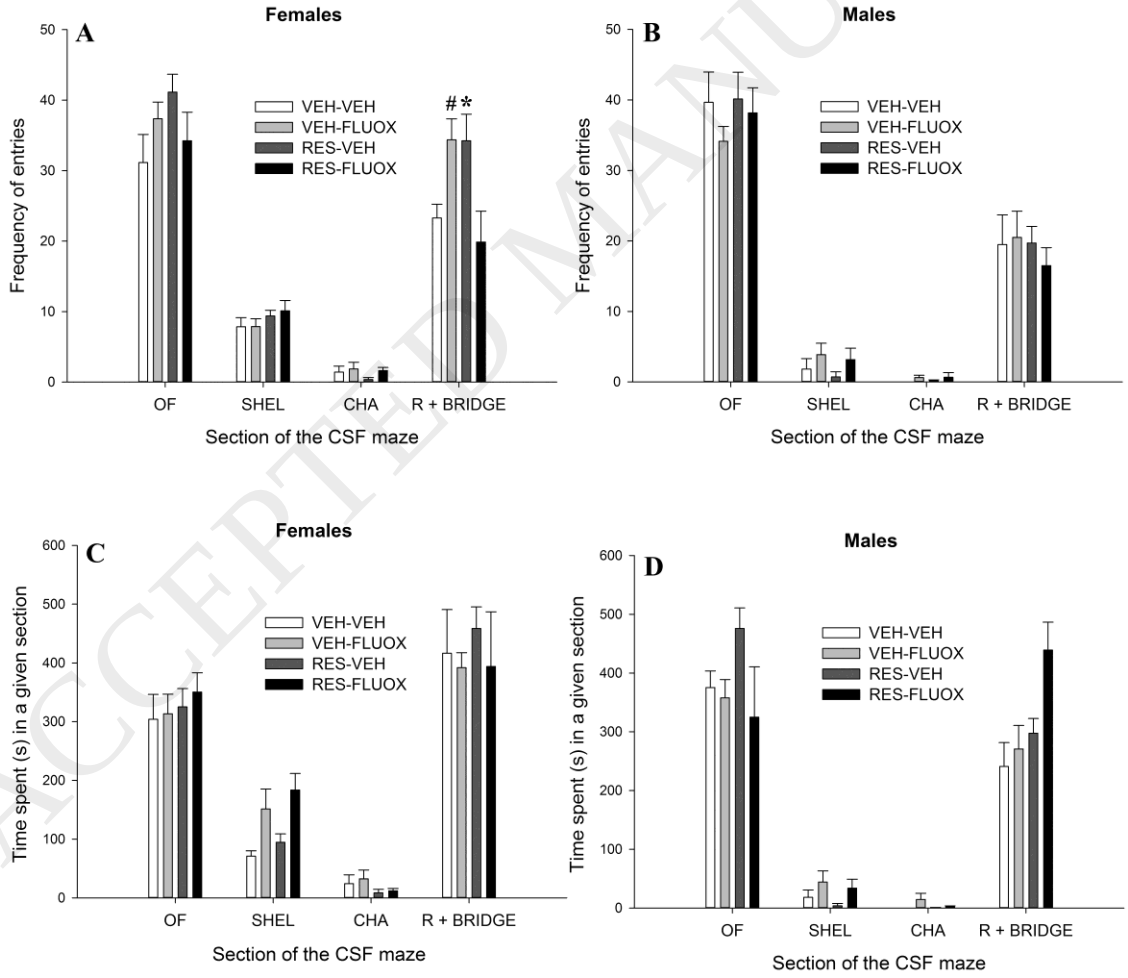

Table 1. Water intake (ml/100g of body weight) and body weight (g) registered at ethanol intake sessions 1 to 5, as a function of reserpine treatment at PDs 30-33 and fluoxetine treatment at PDs 34-37, in male and female rats. Values express mean ± SEM.

	Reserpine treatment at PDs 30-33 and Fluoxetine treatment at PDs 34-37							
	Vehicle (0.0 mg/kg)				Reserpine (1.0 mg/kg)			
Ethanol Intake Session	**Fluoxetine (10 mg/kg)**		Vehicle (0.0 mg/kg)		**Fluoxetine (10 mg/kg)**		Vehicle (0.0 mg/kg)	
	Females	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**
	Water intake (ml/100g of body weight)							
1	4.89 ±1.29	6.27 ±1.04	6.60 ±1.24	7.02 ±1.02	3.06 ±0.78	3.42 ±0.81	4.16 ±0.82	5.66 ±0.59
2	6.04 ±0.97	8.40 ±0.61	8.41 ±0.67	8.95 ±0.61	4.02 ±0.66	4.68 ±0.82	5.08 ±0.84	7.29 ±0.61
3	6.95 ±1.28	9.87 ±0.75	7.83 ±0.54	10.20 ±0.38	3.78 ±0.87	6.91 ±0.72	6.10 ±0.74	7.86 ±1.11
4	8.89 ±0.54	9.14 ±0.51	10.43 ±0.68	9.27 ±0.50	5.26 ±0.80	7.40 ±0.63	6.48 ±0.95	7.98 ±0.76
5	9.84 ±0.47	9.17 ±1.11	10.44 ±0.59	10.23 ±0.66	5.70 ±0.90	7.29 ±1.37	7.72 ±0.68	9.74 ±0.67
	Body weight (g)							
1	116.77 ±2.76	134.00 ±4.75	111.22 ±2.20	132.11 ±4.01	103.88 ±2.26	119.11 ±6.32	102.89 ±4.23	122.30 ±6.78
2	114.78 ±2.63	127.56 ±6.32	110.00 ±2.20	125.66 ±4.86	100.75 ±2.12	114.44 ±7.67	101.11 ±4.31	116.00 ±8.04
3	113.56 ±2.79	128.67 ±6.56	110.11 ±2.42	129.44 ±5.64	98.88 ±2.16	113.11 ±8.66	100.56 ±4.26	116.00 ±8.22
4	112.89 ±2.57	134.78 ±4.81	111.11 ±2.63	136.78 ±4.53	97.50 ±2.58	118.11 ±7.05	100.89 ±4.15	122.80 ±6.82
5	114.33 ±2.58	138.33 ±4.83	112.22 ±2.64	139.78 ±4.14	96.50 ±3.02	119.56 ±6.90	101.11 ±4.07	125.50 ±6.75