THE ROLE OF CRF AND CRF RECEPTORS IN THE EFFECTS OF ALCOHOL INTOXICATION AND WITHDRAWAL

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CONTENTS

PUBLICATIONS

1. Original publications the present work was based on:

1.1. **Simon B**, Buzás B, Bokor P, Csabafi K, Ibos KE, Bodnár É, Török L, Földesi I, Siska A, Bagosi Z: The effects of alcohol intoxication and withdrawal on hypothalamic neurohormones and extrahypothalamic neurotransmitters (Biomedicines. 2023 Apr 27; 11:1288) **Rank: Q1, impact factor: 4.757**, **citation: 0**

1.2. **Simon B**, Thury AÁ, Török L, Földesi I, Csabafi K, Bagosi Z: The effects of alcohol immediately and a day after binge drinking (Alcohol. 2023 May 24; 112:17-24). **Rank: Q2, impact factor: 2.558, citation: 0**

2. Original publications related to the present work:

2.1. Bagosi Z, Palotai M, **Simon B**, Bokor P, Buzás A, Balangó B, Pintér D, Jászberényi M, Csabafi K, Szabó G. Selective CRF2 receptor agonists ameliorate the anxiety- and depression-like state developed during chronic nicotine treatment and consequent acute withdrawal in mice (Brain Research, 2016 Dec 1; 1652:21-29.). **Rank: Q1, impact factor: 2.746, citation: 16**

2.2. Buzás A, Bokor P, Balangó B, Pintér D, Palotai M, **Simon B**, Csabafi K, Telegdy G, Szabó G, Bagosi Z. Changes in striatal dopamine release and locomotor activity following acute withdrawal from chronic nicotine are mediated by CRF1, but not CRF2, receptors (Brain Research, 2019 Mar 1; 1706:41–47.). **Rank: Q2**, **impact factor: 3.125, citation: 6**

2.3. Pintér D, Balangó B, **Simon B**, Palotai M, Csabafi K, Dobó É, Ibos KE, Bagosi Z: The effects of CRF and the urocortins on the hippocampal acetylcholine release in rats (Neuropeptides. 2021 Aug 1; 88:102147). **Rank: Q2**, **impact factor: 3.286, citation: 1**

3. Conference presentations related to the present work:

3.1. **Simon B,** Palotai M, Bagosi Z: The effects of binge drinking on anxiety-like, depression-like and social behaviors (HMAA, Balatonfüred, Hungary, 2019)

4. Poster presentations related to the present work:

4.1. Bagosi Z, **Simon B**, Jászberényi M, Szabó G: The effects of nicotine withdrawal on the rat limbic system: increase of dopamine release in the striatum and increase of GABA release in the amygdala (MÉT, Szeged, Hungary, 2010)

4.2. Bagosi Z, Palotai M, **Simon B**, Bokor P, Buzás A, Csabafi K, Szabó G: The effects of a selective CRFR1 antagonist in rats exposed to chronic nicotine treatment and consequent acute withdrawal (IBRO, Budapest, Hungary, 2016)

4.3. Bagosi Z, **Simon B**, Karasz G, Csabafi K, Ibos K, Szakács J, Ibos KE, Szabó G: Binge drinking and hangover have different impacts on mood (FAMÉ, Budapest, Hungary, 2019)

4.4. Bagosi Z, **Simon B**, Karasz G, Csabafi K, Ibos K, Szakács J, Ibos KE, Szabó G: Binge drinking has different effects on sociability and preference for social novelty (FEPS, Bologna, Italy, 2019)

4.5. Bagosi Z, **Simon B**, Karasz G, Ibos KE, Dobó É, Csabafi K: Different effects of binge drinking and hangover on mood are mediated by different CRF receptors (IBRO, Szeged, Hungary, 2020)

Total impact factors: 16.642
Total citations: 23 (without self-citation: 19)

ABBREVIATIONS

[^{3}H] = tritium

ACTH = adrenocorticotropic hormone

AVP = arginine vasopressin

BNST = bed nucleus of stria terminalis

CEA = central nucleus of the amygdala

CNS = central nervous system

CORT = corticosterone

CRF = corticotropin-releasing factor, also known

CRF-BP = corticotropin-releasing factor-binding protein

CRF1 = corticotropin-releasing factor receptor type 1

CRF2 = corticotropin-releasing factor receptor type 2

CRH = corticotropin-releasing hormone

DA = dopamine

GABA = gamma aminobutyric acid

GLU = glutamate

GPCR = G protein-coupled receptor

HPA = hypothalamic-pituitary-adrenal

IBD = inflammatory bowel disease

IBS = irritable bowel syndrome

ICV = intracerebroventricular

IP = intraperitoneal

LC = locus coeruleus

NACC = nucleus accumbens

PTSD = post-traumatic stress disorder

PVN = paraventricular nucleus

SCP = stresscopin

SNS = sympathetic nervous system

SRP = stresscopin-related peptide

UCN1 = urocortin 1

UCN2 = urocortin 2

UCN3 = urocortin 3

VTA = ventral tegmental area

1. INTRODUCTION

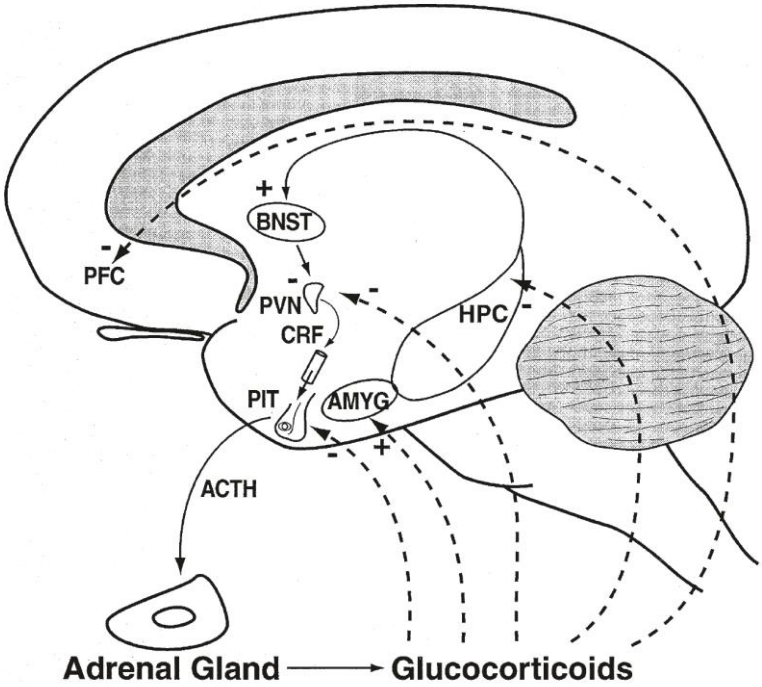
1.1. CRF and CRF receptors

Corticotropin-releasing factor (CRF), also known as corticotropin-releasing hormone (CRH), is a 41 amino acid neuropeptide that acts as a hypothalamic neurohormone, but also as an extrahypothalamic neurotransmitter [1, 2] (**Figure 1**). The major role CRF is to mediate the neuroendocrine, autonomic and behavioral responses to stress [1, 2]. The neuroendocrine stress response is represented by the activation of the hypothalamic-pituitary-adrenal (HPA) axis that is initiated by CRF and/or the synergistic arginin vasopressin (AVP) released from the paraventricular nucleus (PVN) of the hypothalamus that stimulate the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary and subsequently the release of glucocorticoids from the adrenal cortex, that consist mainly from corticosterone (CORT) in rodents and cortisol in humans [1, 2]. The elevation of the plasma ACTH and glucocorticoid levels, not only reflects the activation of the HPA axis, but also exerts a negative feedback effect on the release of hypothalamic CRF and/or AVP, thereby it inhibits the HPA axis [1, 2]. The autonomic stress response is represented by the activation of the sympathetic nervous system (SNS) that is mediated by noradrenaline released from the medulla oblongata and adrenaline from the adrenal medulla [1, 2]. The behavioral stress response is manifested by increased locomotor activity in a familiar environment, decreased locomotor activity in an unfamiliar environment, decreased food and water intake, decreased social and sexual interactions [1, 2].

The effects of CRF are mediated through two different CRF receptors, CRF1 and CRF2 [3, 4]. CRF1 and CRF2 belong to the class B subtype of G protein–coupled receptor (GPCR) superfamily having 415 and 397-437 amino acids, respectively. They have several splice variants [5]: CRF1 has α and β isoforms in addition to subtypes designated c-h, which have been detected in rodent and human tissues [6], whereas CRF2 has three functional subtypes, α, β, and γ [6]. CRF2α and CRF2β have been detected in rodents, primates and humans, while CRF2γ has only been reported in humans [6].

The original hypothesis states that CRF1 and CRF2 promote dualistic effects in the CNS, with CRF1 initiating the stress responses, and CRF2 terminating these responses [1, 2, 8] (**Figure 2**). However, a recent hypothesis pretends that the role of CRF1 and CRF2 in stress response is not a matter of simple dualism, but it depends upon the brain regions and neuron populations being activated [7, 8] (**Figure 3**).

A.



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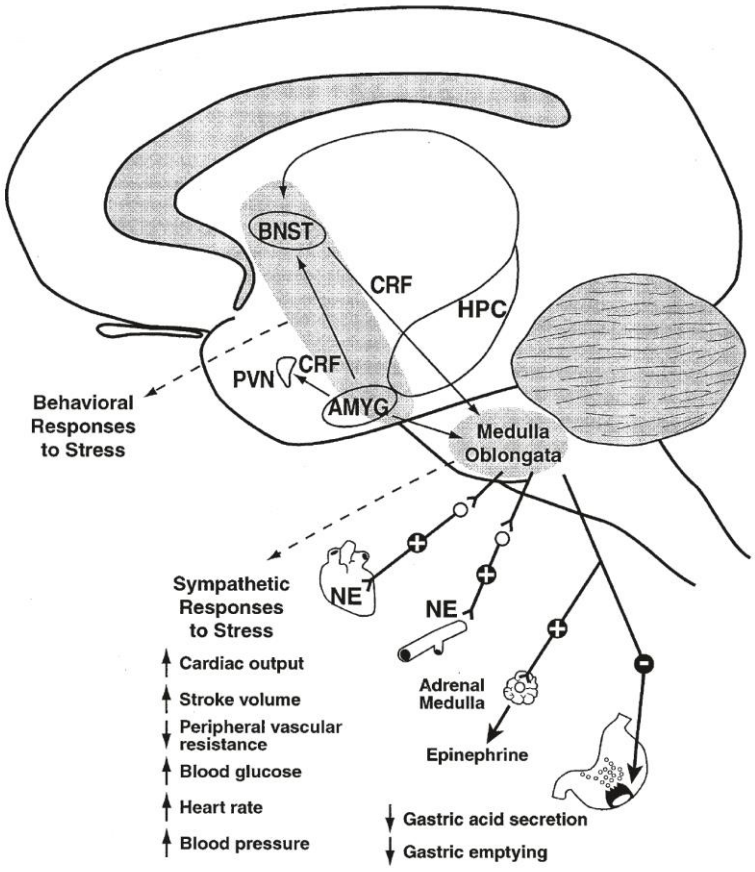


Figure 1. The role of the hypothalamic (A) and extrahypothalamic CRF (B) [9]

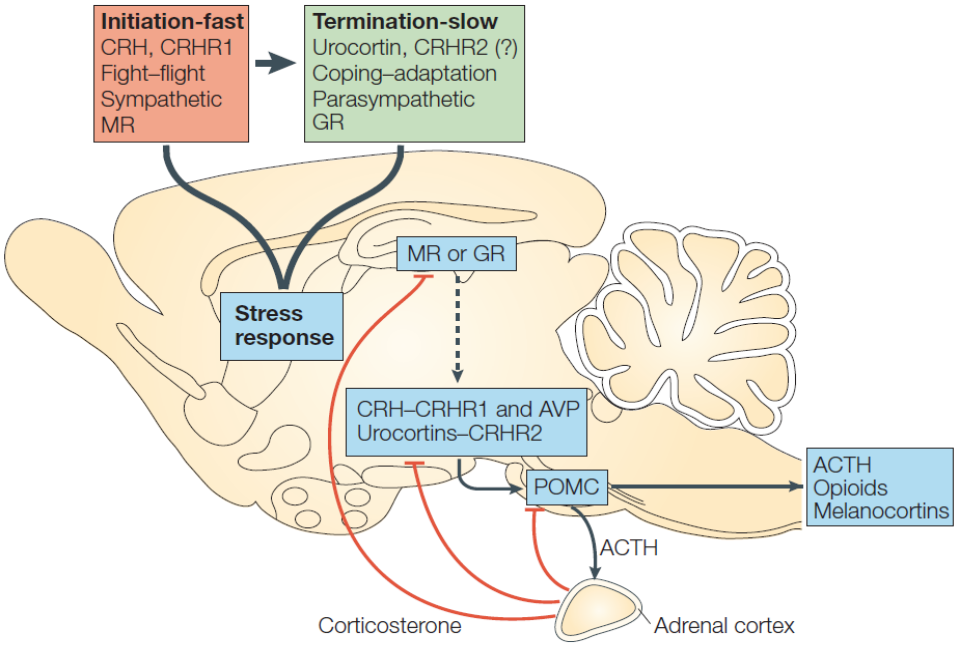
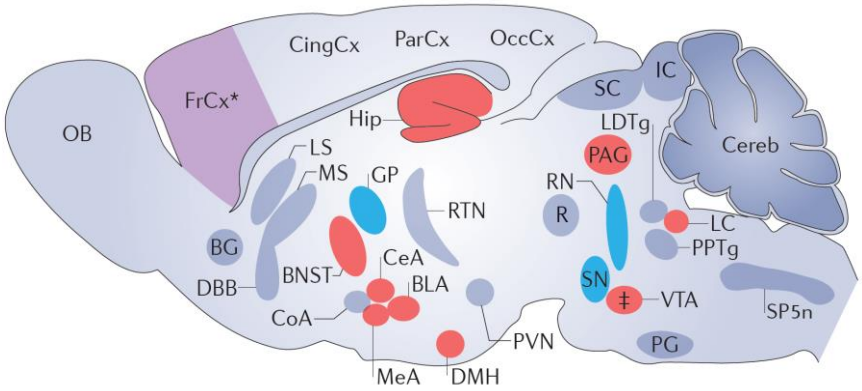


Figure 2. The dualistic role of CRF1 and CRF2 in stress response [10]

A.



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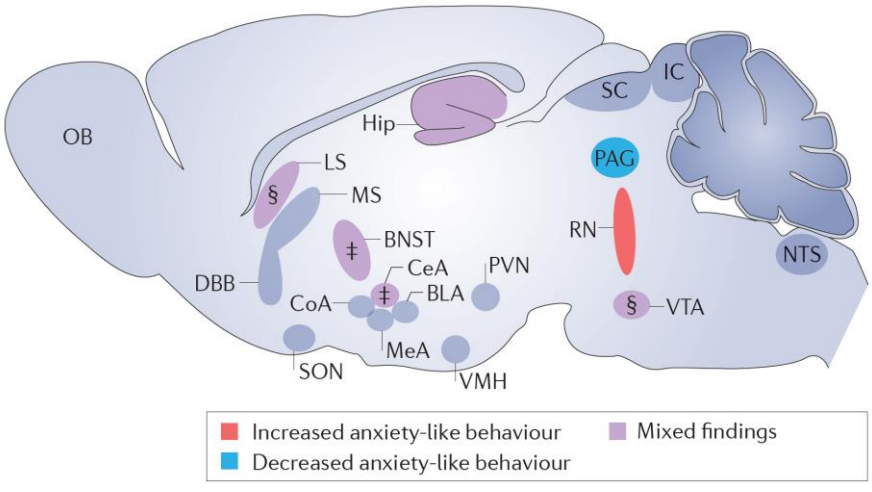


Figure 3. The region-specific role of CRF1 (A) and CRF2 (B) in stress response [8]

Since corticotropin-releasing factor (CRF) was first isolated in 1981, a growing family of CRF-like peptides, called the urocortins, has been discovered [11]. Despite of the different anatomical distribution and physiological functions, CRF and the urocortins share similar amino acidic sequences and activate similar intracellular pathways [11] (**Figure 4**).

A.

Peptide	Sequence	Length	Identity (%)
hCRF	SEEPPISLDLTFHLLREVLEMARAEQLAQQAHSNRKLMEII	41	100
oCRF	SQEPPISLDLTFHLLREVLEMTKADQLABQAHSNRKLLDIA	41	83
URO	NDDPPISIDLTFHLLRNMIEMARIENEREQAGLNRKYLDEV	41	54
hUCN	DNPSLSIDLTFHLLRTLLELARTQSQRERAEQNRIIFDSV	40	43
SVG	ZGPPISIDLSLELLRKMIEIEKQEKEKQQAANNRLLLDTI	40	48
hSRP	IVLSLDVPIGLLQILLEQARARAAREQATTNARILARV	38	34
mUCNII	VILSLDVPIGLLRILLEQARYKAARNQAATNAQILAHV	38	34
hSCP	FTLSLDVPTNIMNLLFNIAKAKNLRAQAAANAHLMAQI	38	32
mUCNIII	FTLSLDVPTNIMNILFNIDKAKNLRAKAAANAQLMAQI	38	26

B.

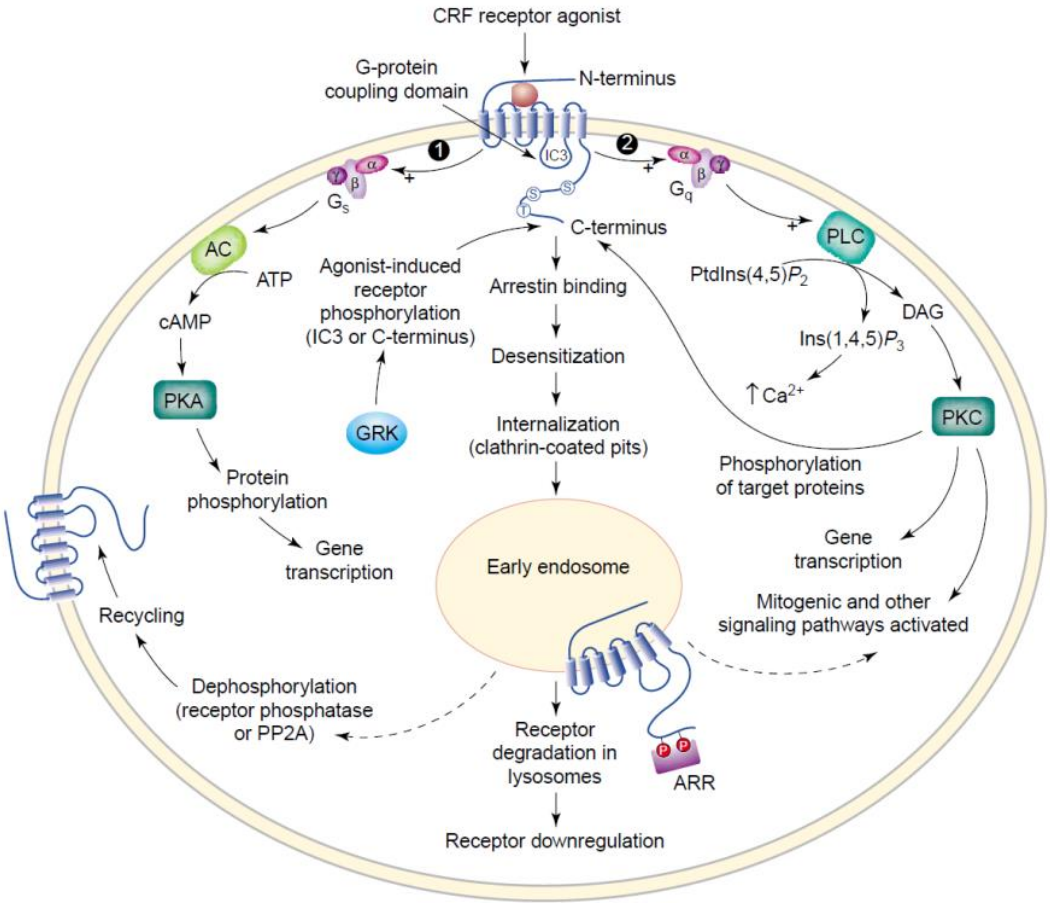


Figure 4. The amino acidic sequences shared (A) and intracellular pathways activated (B) by CRF and the urocortins [11]

Urocortin 1 (UCN1) is a 40 amino acid neuropeptide that was isolated first from rat brain in 1995 [12]. The name urocortin derives from the fish urotensin (63% homology) and the mammalian corticotropin(-releasing factor) (45% homology) [11]. In the rodent brain, UCN1 is expressed predominantly in the Edinger-Westphal nucleus (EWN). This nucleus is usually involved in the oculomotor, pupillary and auditory functions, but, because of its UCN1 expression, it is probably implicated in the neuroendocrine and behavioral stress responses, as well [13-15] (**Figure 5**).

Urocortin 2 (UCN2) is a 38 amino acid neuropeptide that was identified first in mouse brain in 2001 [16]. In humans, it is also known as stresscopin-related peptide (SRP) (34% homology with CRF) [11]. The abundant expression of UCN2 in different nuclei of the hypothalamus and locus coeruleus (LC) suggests its role in the behavioral and autonomic stress responses, including regulation of food and water intake, and locomotion [13-15].

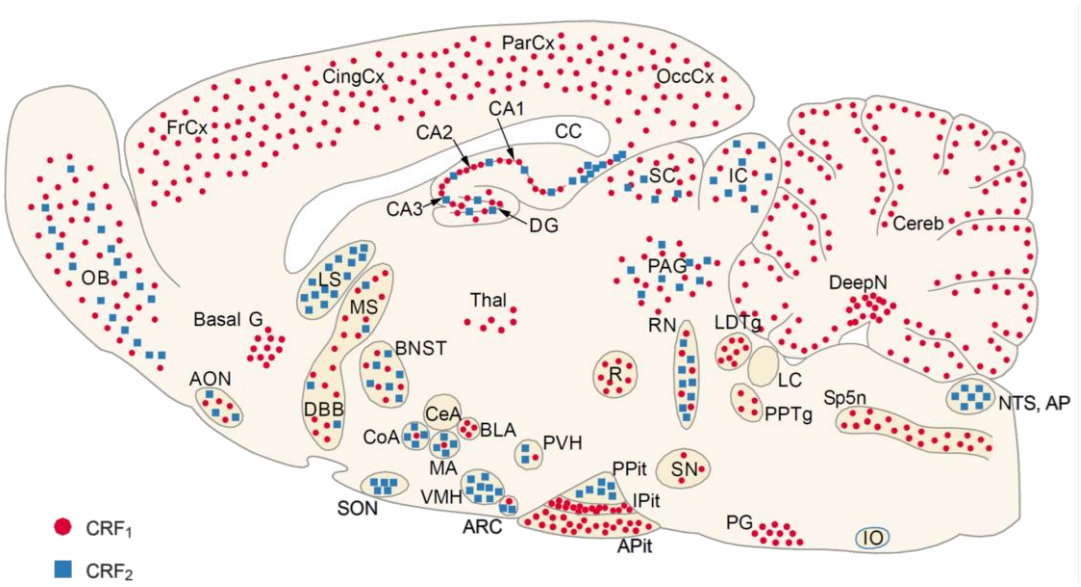
Urocortin 3 (UCN3) is another 38 amino acid neuropeptide identified in the mouse brain in 2001 [17]. In humans, it is also known as stresscopin (SCP) (36% homology with CRF) [11]. The abundant expression of UCN3 in brain regions that are closely related or reciprocally connected with the PVN, suggests that it contributes to the neuroendocrine and behavioral adaptation to stress, including regulation of food and water intake, and locomotion [13-15].

Just like CRF, the urocortins bind to CRF1 and CRF2. Both CRF receptors are found in the CNS and the periphery, although CRF1 is expressed more abundantly in the central nervous system (CNS), whereas CRF2 is more dominant in the periphery [13]. In the CNS, CRF1 is distributed abundantly the cerebral cortex, cerebellum, and anterior pituitary [11], whereas CRF2 is limited to subcortical regions, such as the hypothalamus, hippocampus, amygdala, and posterior pituitary [13].

As regards their pharmacological profile, while CRF binds with higher affinity to CRF1 than CRF2, and acts preferentially through CRF1 [14], UCN1 binds with higher affinity to CRF1 and CRF2 than CRF itself, and activates both CRF receptors equipotently [14]. Hence, CRF and UCN1 are considered non-selective agonists of CRF1 [15]. In addition, CRF and UCN1 can be bound by CRF-binding protein (CRF-BP) [18], a 322 amino acid protein that is believed to inhibit the effects of CRF and UCN1 [16, 17]. UCN2 and UCN3 have a much higher affinity for CRF2, than CRF1, and especially UCN3 activates selectively CRF2 [13]. Since UCN2 and UCN3 cannot be bound by CRF-BP either, they are considered selective agonists of CRF2 [18] (**Figure 6**).

As regards their physiological functions, activation of CRF1 by administration of CRF and UCN1 induced activation of the HPA axis, anxiety-like and depression-like behavior [2, 17, 18], whereas activation of CRF2 by administration of UCN2 and UCN3 produced anxiolytic and antidepressant effects in rats [19-22] (**Figure 7**). However, overexpression of CRF and global and site-specific knock-out of CRF1 and CRF2 in mice led to different results regarding anxiety-like behavior (**Figure 8**). Therefore, the exact role of CRF1 and CRF2 in the activation of the HPA axis, anxiety and depression is still under debate [24-28].

A.



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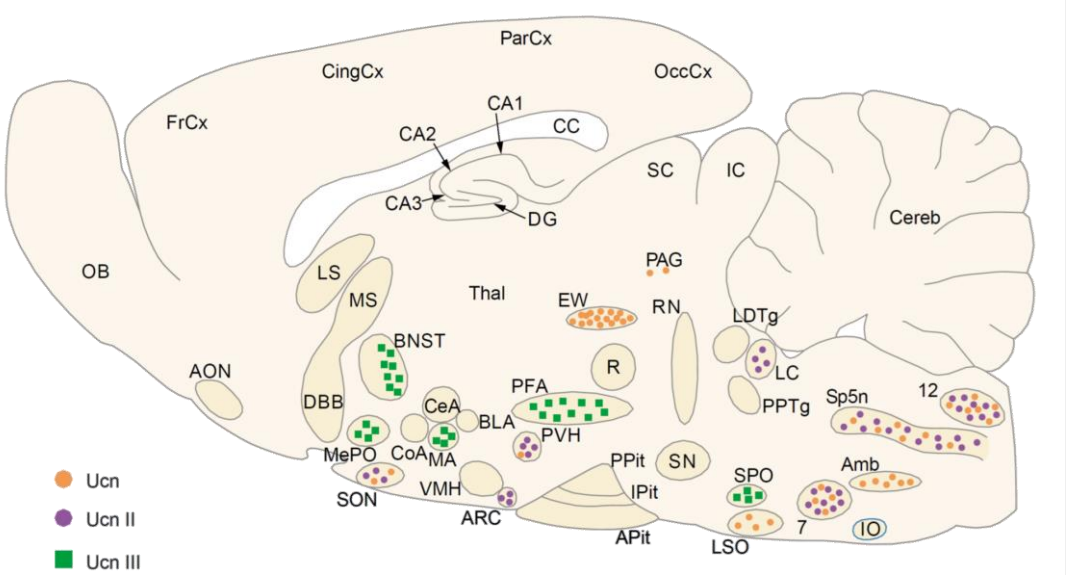

Figure 5. The anatomical distribution of CRF and the urocortins (A) and CRF receptors (B) [13]

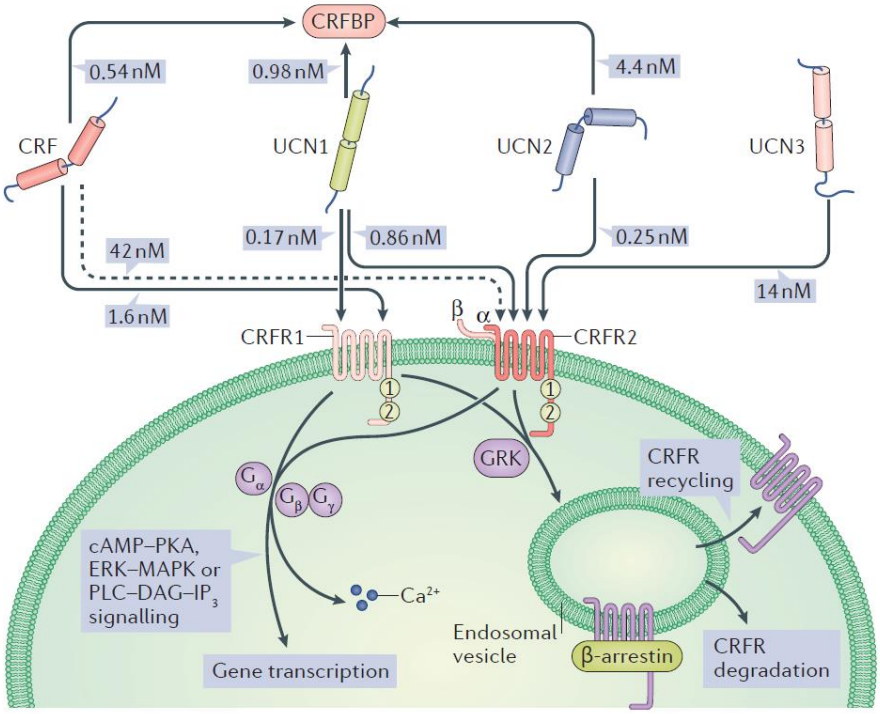

Figure 6. The pharmacological profile of CRF and the urocortins [8]

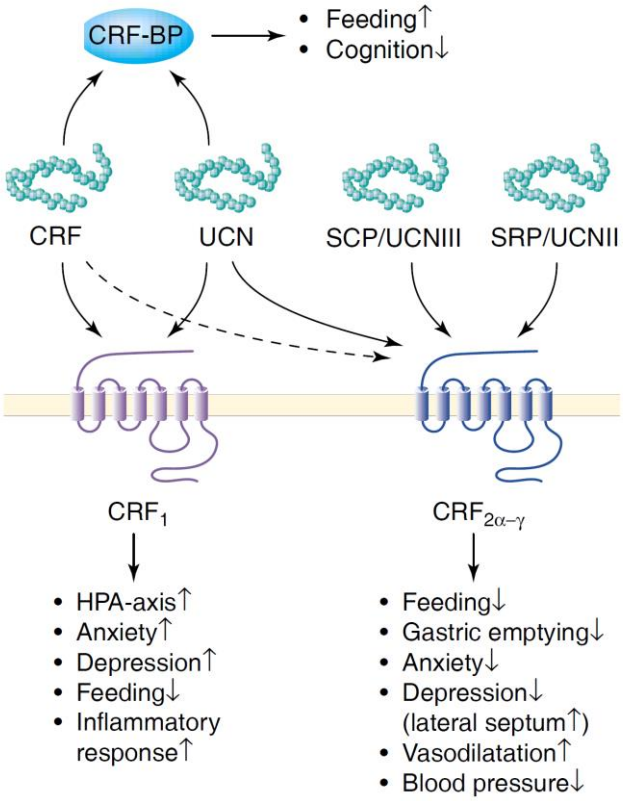

Figure 7. The physiological functions of CRF and the urocortins [11]

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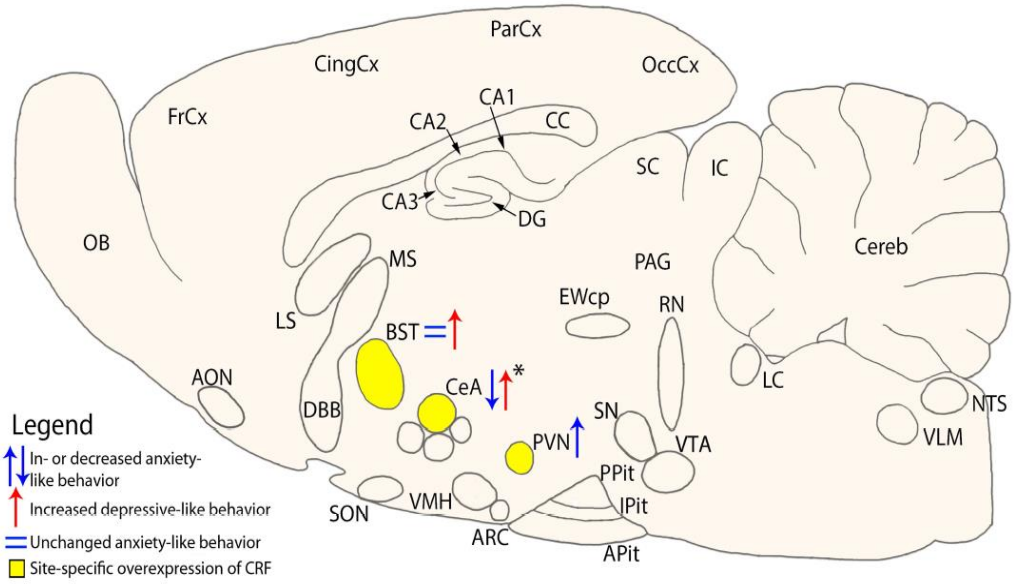

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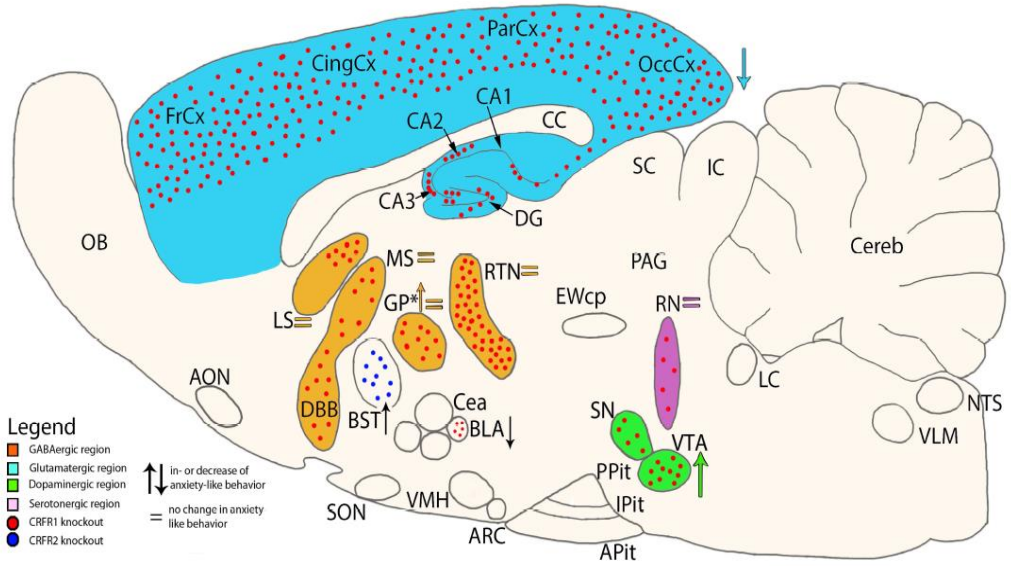


Figure 8. The overexpression of CRF (A) and global and site-specific knock-out of CRF1 and CRF2 (B) [7]

In order to determine the exact role of CRF1 and CRF2 in the activation of the HPA axis, anxiety and depression CRF receptor antagonists were used in animal experiments (**Figure 9**). First, non-selective CRF antagonists, such as α-helical CRF 9-41 and D-Phe CRF were developed. These are peptidic, competitive, but non-selective CRF receptor antagonists, which blocked efficiently the ACTH release and locomotor activation induced by CRF and stress [19-21]. The next was astressin that was particularly potent at inhibiting the HPA axis and reducing anxiogenic-like behavior, but failed to reverse the locomotor hyperactivity induced by CRF and stress [22]. The first truly selective antagonists were CP-154,526 and its structural analog antalarmin [23-27]. These are non-peptidic, competitive and selective CRF1 antagonists, which were could attenuate stress, anxiety-like and depression-like behavior in rodents [23-27]. Therefore, it was suggested that selective CRF1 antagonists, such as Pexacerfont and Verucerfont, along with selective CRF2 agonists, such as UCN2 and UCN3 could be used to treat stress-related psychiatric disorders, including anxiety, depression, post-traumatic stress disorder (PTSD), and panic disorder**.** Next, selective antagonists of CRF2, such as antisauvagine-30 and astressin2B were developed, these were derived from the frog analog sauvagine and astressin, respectively [19, 28-31]. Antisauvagine and astressin2B are peptidic, competitive and selective CRF2 antagonists, which are usually administered peripherally [19, 28-31]. Since CRF1 increases colonic transit, while CRF2 decreases gastric empyting, it was suggested that selective CRF1 agonists, such as Stressin1-A and selective CRF2 antagonists, such as antisauvagine and astressin2B can be used to ameliorate stress-induced gastrointestinal diseases, including irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) [28] **(Figure 10)**.

Unfortunately, these drugs that seemed promising in animal experiments, were ineffective in humans [32]. This could be due to the fact that when neuropeptides are administered orally, they are degraded by enzymes in the gastrointestinal tract, and when they are administered intravenously, they are unable to cross the blood-brain barrier. However, there are still some ongoing clinical trials that may prove the efficacy of CRF1 antagonists in the therapy of alcohol addiction [33].

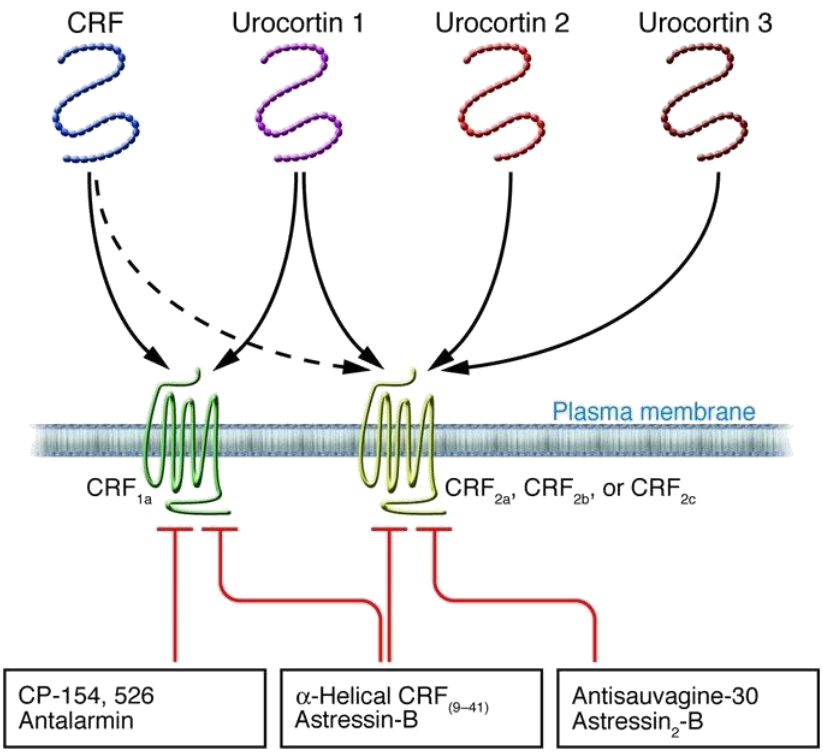


Figure 9. The experimental use of CRF receptor antagonists [34]

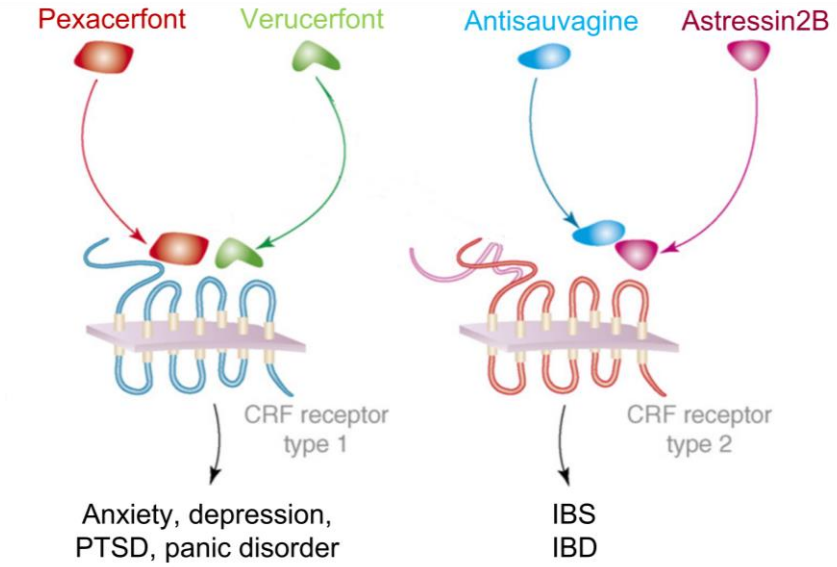


Figure 10. The therapeutical use of CRF receptor antagonists [35]

1.2. Alcohol intoxication and withdrawal

Alcohol addiction, termed lately alcohol use disorder, is a chronically relapsing disorder that is characterized by the compulsion to seek and take the drug, the loss of control in limiting intake, and the emergence of a negative emotional state when access to the drug is prevented [36, 37]. Alcohol addiction has three stages: binge or intoxication, withdrawal or negative affect, and craving or preoccupation and anticipation [36, 37]. Each stage is characterized by specific changes of hypothalamic neurohormones, such as CRF and AVP, and extrahypothalamic neurotransmitters, such as striatal dopamine (DA), amygdalar gamma aminobutyric acid (GABA) and hippocampal glutamate (GLU) [36, 37] (**Figure 11**).

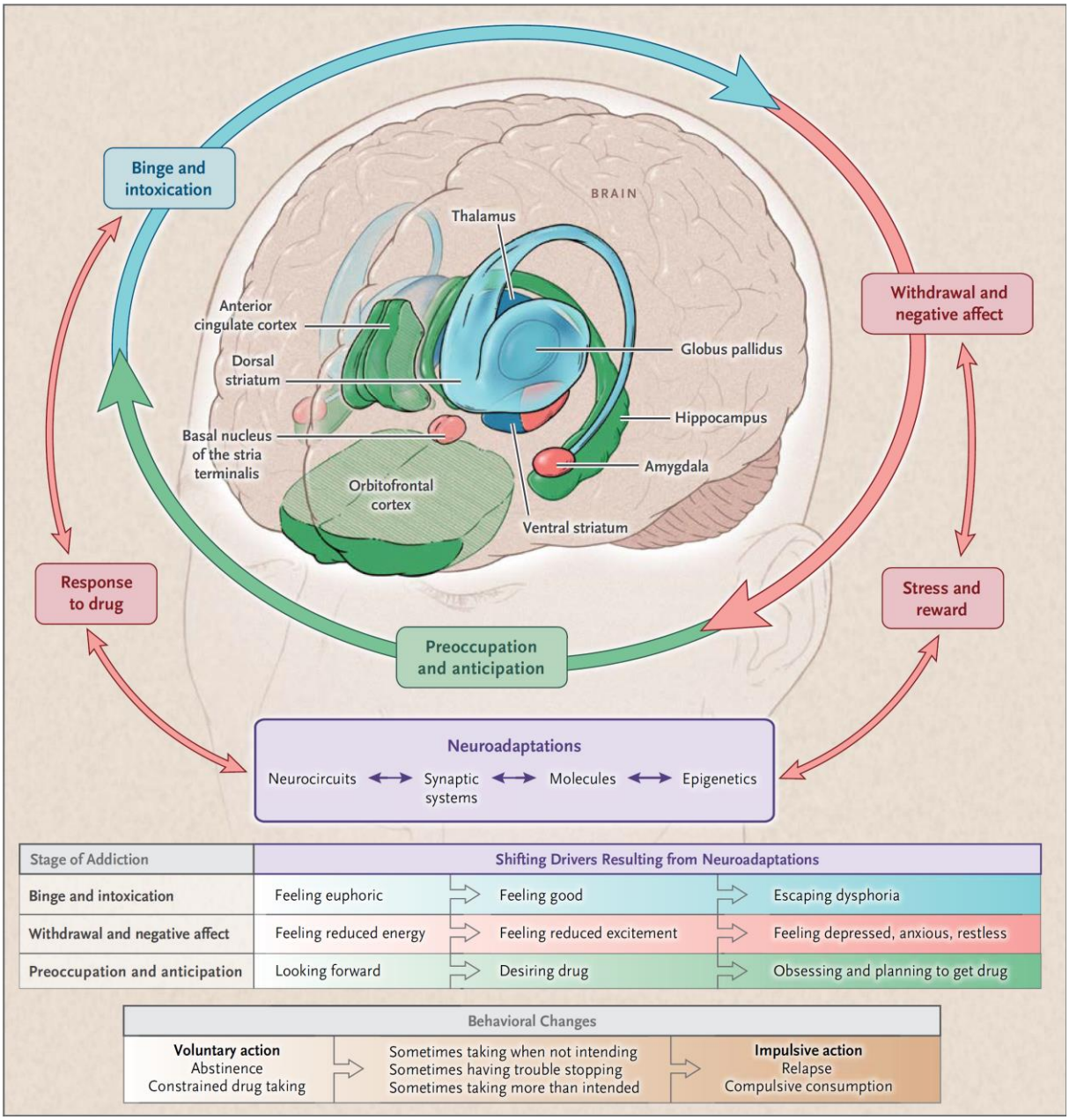


Figure 11. Behavioral changes in the stages of alcohol addiction [38]

Binge drinking itself is defined as consuming a large amount of alcohol in a short period of time [39]. A large amount of alcohol refers to five or more standard alcoholic drinks in men and four or more standard alcoholic drinks in women (**Figure 12**) that, by definition, brings their blood alcohol concentration (BAC) to 0.08 g/dL within 2 hours and is commonly associated with acute impairment in motor coordination and cognitive functioning [39]. Hangover is a temporary state described as the unpleasant next-day effects after binge drinking [40]. This state usually emerges after a single episode of heavy drinking when BAC approaches zero and is associated with a combination of physical signs, such as ataxia, locomotor and exploratory dysfunctions, and affective symptoms, such as fear, anxiety, and depression [40]. Alcohol addiction is best resembled by alternating episodes of binge drinking and hangover [41, 42]. Individuals who regularly engage in episodic heavy drinking do not entirely meet the diagnostic criteria for alcohol use disorder, however repeating cycles of binge drinking that emerge during adolescence is an important risk factor for development of alcohol addiction in adulthood [41, 42]. Repeating episodes of binge drinking may elicit persistent negative affect, including anxiety and depression [42-45], and alteration of social behavior similar to that described during acute alcohol withdrawal [46, 47]. Nevertheless, anxiety, depression and hangover are usually attributed to humans. In the present study we used male Wistar rats and C57BL/6 mice, hereby we refer to these terms as anxiety-like and depression-like signs or behavior, and hangover-like symptoms.

Figure 12. A standard alcoholic drink

The stage of binge or intoxication is associated with the activation of the HPA axis that is initiated by hypothalamic CRF and/or AVP and the activation of the mesolimbical and nigrostriatal dopaminergic pathways [43, 44] (**Figure 13**). The HPA axis is represented by the paraventricular nucleus of the hypothalamus (PVN), the anterior pituitary and the adrenal cortex, and can be stimulated by various stressors, including alcohol intoxication [45, 46]. The mesolimbical pathway sends dopaminergic projections from the ventral tegmental area (VTA) to the nucleus accumbens (NACC), that represents the ventral striatum, whereas the nigrostriatal dopaminergic pathway projects from the substantia nigra to the putamen and nucleus caudatus, which represent the dorsal striatum [43].

The stage of withdrawal or negative affect is associated with the activation of the extended amygdala circuit that is mediated by extrahypothalamic CRF and noradrenaline [43]. The extended amygdala circuit consists of the central nucleus of the amygdala (CEA), the bed nucleus of stria terminalis (BNST), and the shell of the nucleus accumbens (NACC) and represents an interface between reward and stress systems [45, 46]. During alcohol intoxication the reward system is activated [43]. Alcohol stimulates the dopamine (DA) release in the striatum, and the gamma aminobutyric acid (GABA) release in the amygdala, inducing rewarding, anxiolytic and antidepressant effects [43]. Alcohol intoxication may also result in amnesia that can be related to the reduction of the hippocampal glutamate (GLU) release [43]. During alcohol withdrawal, as an anti-reward mechanism, the stress system is activated, resulting in alcohol withdrawal syndrome that includes symptoms, such as anhedonia, anxiety and aggression [43]. These symptoms can be explained by the reduction of the striatal DA and amygdalar GABA release, and the stimulation of the GLU release in the hippocampus [43]. The alcohol withdrawal syndrome consists of somatic (physical) signs and affective (emotional) symptoms that emerge immediately after alcohol cessation [36, 37]. The physical signs usually cease within 24 hours following alcohol intoxication (acute alcohol withdrawal), whereas the emotional symptoms may persist for days to years (protracted alcohol withdrawal), producing craving that makes one vulnerable to relapse, especially in periods of stress [36, 37]. This last stage of preoccupation/anticipation (craving) is associated with the activation of the hippocampus, orbitofrontal cortex, prefrontal cortex, insula and basolateral amygdala and believed to be mediated by both hypothalamic and extrahypothalamic CRF [43].

A.

B.

C.

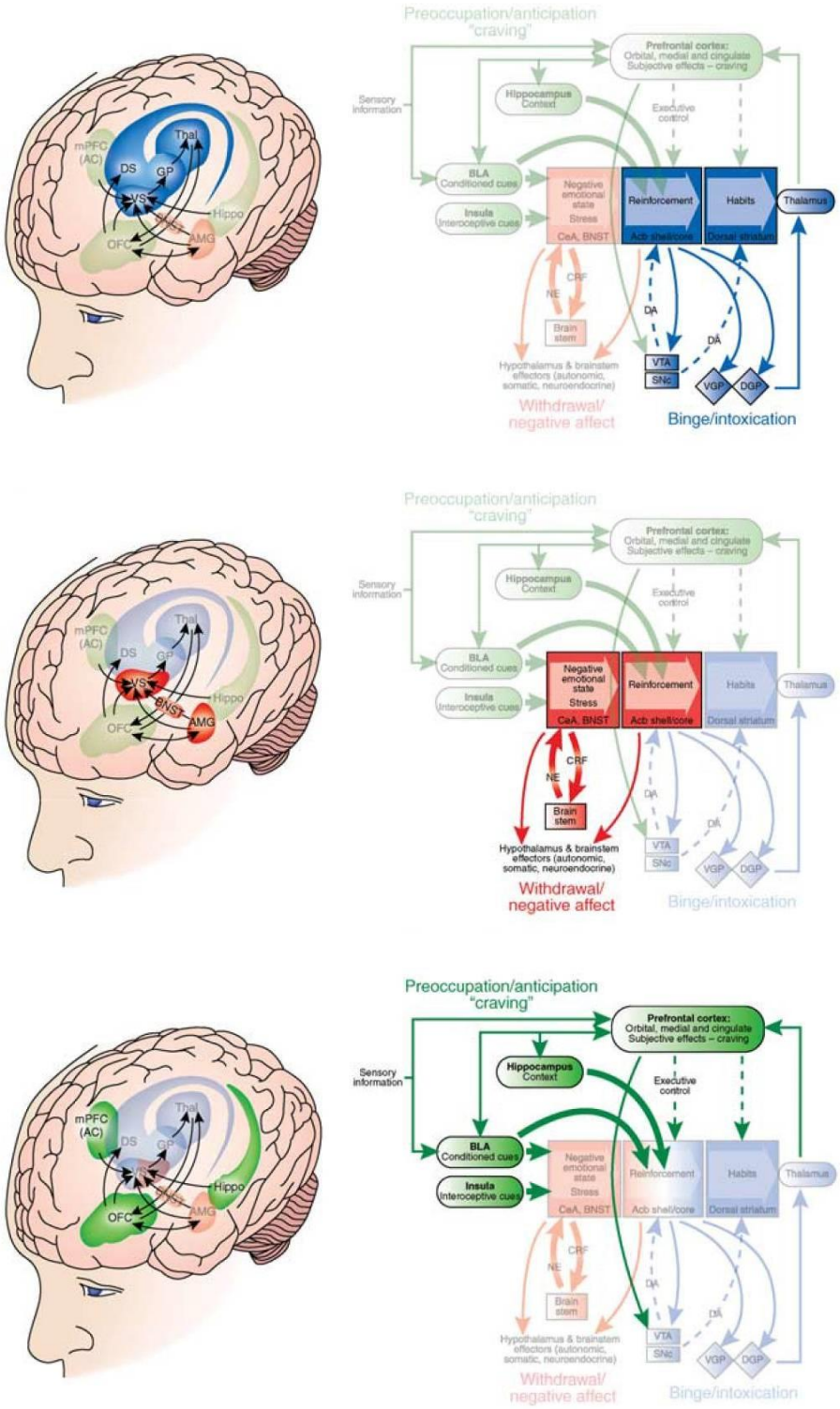


Figure 13. Neuroendocrine changes in the stages of alcohol addiction: binge or intoxication (A), withdrawal or negative affect (B) and craving or preoccupation and anticipation (C) [43]

2. AIM OF STUDY

The first aim of the present study was to determine the effects of binge drinking and hangover on anxiety-like, depression-like and social behavior. For this purpose, male C57BL/6 mice were exposed to 4 days of drinking in the dark (a classical method to investigate binge drinking in animals) and 1 day of withdrawal. Since the CRF and CRF1 have been involved in the pathogenesis of anxiety and depression, and various aspects of social behavior, the participation of the CRF1 and CRF2 in these effects was also investigated by intracerebroventricular (ICV) pretreatment of mice with selective CRF1 antagonist antalarmin and selective CRF2 antagonist astressin2B.

The second aim of the present study was to determine the effects of alcohol intoxication and withdrawal on hypothalamic neurohormones and extrahypothalamic neurotransmitters. For this purpose, male Wistar rats were exposed to repeated intraperitoneal (IP) administration of alcohol for 4 days and then for 1 day of withdrawal. Since CRF has been implicated in all three stages of alcohol addiction, the participation of the CRF receptors was investigated. Therefore, rats were also treated ICV of selective CRF receptor antagonists.

3. MATERIALS AND METHODS

3.1. Animals

Male C57BL/6 mice (Charles River Laboratories Ltd., Hungary) weighing 18-24 g were used. The mice were house together and kept in their home cages at a constant temperature on an inverted illumination schedule with 12-h light and 12-h dark periods (lights on from 18:00, dark on from 6:00), except during drinking in the dark, when they were kept separated in their cage. Commercial food and tap water were available ad libitum, and for a few hours their water bottles were changed with alcohol of 20%. To minimize the effects of non-specific stress the mice were handled daily. All tests were performed between 9:00 to 12:00. Male Wistar rats (Charles River Laboratories Ltd., Hungary) weighing 150-250 g were also used. The rats were housed together and kept in their home cages at a constant temperature on a standard illumination schedule with 12-h light and 12-h dark periods (lights on from 6:00). Commercial food and tap water were available ad libitum. To minimize the effects of non-specific stress the rats were handled daily. All animals were treated in accordance with the ARRIVE guidelines and the experiments were carried out in concordance with the EU Directive 2010/63/EU for animal experiments.

3.2. Surgery

The mice were implanted with a stainless steel Luer cannula, aimed at the right lateral cerebral ventricle under anesthesia with 60 mg/kg pentobarbital sodium (Euthanasol, CEVA-Phylaxia Ltd., Hungary). The stereotaxic coordinates were 0.5 mm lateral and 0.5 mm posterior from the Bregma and 3 mm deep from the dural surface according to the stereotaxic atlas of the mouse brain [47]. Cannulas were secured to the skull with Ferrobond instant glue (Ferrokémia Ltd., Hungary) and they were closed by a metal string between injections. Before the experiments the mice were allowed for 5 days to recover after the surgery. After the experiments 4 μl of dye methylene blue (Reanal Ltd., Hungary) at 1g/100 ml concentration was injected through the cannula to identify the site of injection. Animals without the dye in the lateral cerebral ventricle were discarded.

The rats were implanted with a stainless steel Luer cannula (10 mm long), aimed at the right lateral cerebral ventricle under anesthesia with 35 mg/kg pentobarbital sodium (Euthanasol, CEVA-Phylaxia, Hungary), in order to perform icv administration of the selective CRF antagonists. The stereotaxic coordinates were 0.2 mm posterior and 1.7 mm lateral to the bregma, 3.7 mm deep from the dural surface, according to a stereotaxic atlas of

the rat brain [11]. Cannulas were secured to the skull with dental cement and acrylate. The rats were allowed for 7 days to recover before the experiments.

3.3. Substances

For the drinking in the dark procedure only tap water and alcohol solution of 20% (Reanal Ltd., Hungary) were needed. For intraperitoneal (IP) treatment saline solution (B. Braun Inc., Germany) and alcohol solution of 20% (Reanal Ltd., Hungary) were used. For intracerebroventricular (ICV) pretreatment saline solution (B. Braun Inc., Germany), antalarmin and astressin2B (Sigma-Aldrich Inc., USA) were used. For the behavioral tests only tap water and sodium hypochlorite solution (HIP-TOM Ltd., Hungary) were needed. In contrast, the laboratory tests required several substances, such as commercially available enzymatic kit (Roche Diagnostics, Germany) used to determine the blood alcohol concentration (BAC). *In vitro* homogenization of the hypothalamus required acetic acid (Reanal Ltd., Hungary). For *in vitro* superfusion of the striatum, amygdala and hippocampus, Krebs solution (Reanal Ltd, Hungary) was prepared and tritium-labelled neurotransmitters, including [3H]DA, [3H]GABA and [3H]GLU (Perkin-Elmer Inc., USA), and Ultima Gold scintillation fluid (Perkin-Elmer Inc., USA) were purchased. For the determination of hypothalamic CRF and AVP expression GeneJET RNA Purification Kit and Maxima First Strand cDNA Synthesis Kit (Thermo Scientific Inc., USA) were used. For the determination of hypothalamic CRF and AVP and plasma ACTH concentration Enzyme-linked Immunosorbent Assay (ELISA) Kits (Phoenix Pharmaceuticals Ltd., Germany) were purchased. For the determination of plasma CORT concentration methylene chloride, sulfuric acid and alcohol solution (Reanal Ltd., Hungary) were prepared.

3.4. Treatments

The mice were exposed to drinking in the dark, a classical animal model for binge drinking [48]. First the dark-light cycle of the mice was inverted for 14 days, and then their water bottles were replaced by alcohol of 20% for 4 days (2 hours on the first, second and third day, and 4 hours on the fourth day). On the 4th day (immediately after binge drinking) or on the 5th day (24 hours after binge drinking) mice were treated ICV with the selective CRF1 antagonist antalarmin or the selective CRF2 antagonist astressin$_2$B. The dose of antalarmin was 0.1 μg/2 μl and that of astressin2B was 1 μg/2 μl, as in our previous experiments these doses have been already proved to block effectively the neuroendocrine stress response, without altering the social behavior of animals [49, 50]. After 30 minutes the

animals were investigated in an elevated plus-maze test or a forced swim test for signs of anxiety or depression, respectively. In parallel, a social interaction test was also performed, in order to investigate the sociability and the preference for social novelty of mice. The mice were assigned randomly for one of the treatments on the 4th day or the 5th day, they were not exposed to more than one icv administration in two consecutive days. Also, the mice were assigned randomly for one of the behavioral tests, they were not exposed consequently to elevated plus-maze, forced swim or social interaction test.

The rats were exposed to repeated IP administration of alcohol every 12 hours, for 4 days and then for 1 day of alcohol withdrawal. The protocol of alcohol administration was based on a previous study in which 20% alcohol was administered at dose of 3 g/kg [51]. On the 5th day (immediately after the last ip administration of alcohol) or the 6th day (24 hours after the last ip administration of alcohol) the rats were administered icv with 0.1 μg/2μl of antalarmin or 1 μg/2μl of astressin$_2$B. The doses of CRF1 and CRF2 antagonists were based on our previous studies, which indicated that these doses proved to block most efficiently the activation of the HPA axis and the striatal DA release observed during nicotine withdrawal [52, 53]. After 30 minutes, the mice were decapitated without anesthesia, the brains were removed and the trunk blood was collected. From the brain the expression and concentration of hypothalamic CRF and AVP, and the release of striatal DA, amygdalar GABA and hippocampal GLU were determined. From the trunk blood the concentration of plasma ACTH and CORT were measured.

3.5. Behavioral tests

3.5.1. Elevated plus-maze test

The mice were investigated in an elevated plus-maze test described first by Lister [54]. The elevated plus-maze apparatus consists of a plus-shaped wooden platform elevated at 40 cm from the floor, made-up by four opposing arms of 30 cm × 5 cm. Two of the opposing arms are enclosed by 15 cm-high side and end walls (closed arms), whereas the other two arms have no walls (open arms). The room where the behavioral tests were performed has been darkened, only the central area of the elevated plus-maze has been enlighten with a lamp found at 50 cm from the platform, having a LED bulb of 3.5 Watts that corresponds for 2,230 Lumens. The principle of the test is that open arms are more fear-provoking and the ratio of the times spent in open *vs.* closed arms, or the ratio of the entries into open *vs.* closed arms, reflects the relative safety of closed arms, as compared with the relative danger of open arms. Each mouse was placed in the central area of 5 cm × 5 cm of the maze, facing one of the open

arms. For a 5 minutes period two parameters were recorded by an observer sitting at 100 cm distance from the center of the plus-maze: the number of entries into the open arms relative to the total number of entries and the time spent in the open arms relative to the total time. All parameters were expressed as percentages. The platform of the apparatus was cleaned with sodium hypochlorite solution between the subjects.

3.5.2. Forced swim test

The mice were also investigated in a forced swim test described first by Porsolt [55]. The forced swim apparatus consists of a plexiglass cylinder of 40 cm height and 12 cm diameter positioned on a table. The cylinder was half-filled with water maintained at 25±1 °C. The principle of the test is that in such a situation, from which they cannot escape, animals rapidly became immobile, that is, floating in an upright position and making only small movements to keep their heads above water. Meanwhile their attempt to escape the cylinder by climbing or swimming may decrease or cease eventually. Each mouse was placed individually into the water. For a 5 minutes period the following parameters were recorded by an observer sitting at 100 cm distance from the table: the time that mice spent with swimming and climbing the walls, in their attempt to remain at the surface and escape the cylinder, respectively, and the time spent with immobility or floating. All parameters were expressed in time units, a time unit consisting of 5 seconds. The water from the cylinder was completely changed between the subjects.

3.5.3. Social interaction test

The mice were also tested in a social interaction arena invented by Crawley [56]. The arena is represented by a rectangular plexiglass box divided into three-chambers, each chamber having size of 19 x 45 x 25 cm. The right and left chambers could be isolated from the middle one by using two dividing plexiglass walls. Two identical, wire cup-like cage of 10 x 17 cm with removable lids that large enough to hold a single mouse were placed vertically inside the apparatus, one in each side chamber. Each cage was comprised of metal wires to allow for air exchange between the interior and exterior of the cylinder but small enough to prevent direct physical (aggressive or sexual) interactions between an animal on the inside with one on the outside. Two types of test were performed: the first test was meant to measure the sociability, whereas the second test was meant to measure the preference for social novelty of the mice [57]. In the first test the tested mouse was first habituated with the middle chamber for 5 minutes and then allowed to explore the remaining chambers for

another 5 minutes: a stranger male mouse in a cage was placed in one of the chambers and an an empty cage was put in the other chamber. The principle of the first test is based on the observation that a wild type mouse enters and spends more time in interaction with the stranger mouse over a foreign object (e.g. empty cage), indicative of intact sociability. In the second test a stranger male mouse in a cage was placed in one of the chambers and the male mouse that was the stranger in the previous test (now considered familiarized) in a cage was placed in the opposite chamber. The principle of this second test is based on the assessment that a wild type mouse enters and spends more time in interaction with the stranger mouse over the familiarized one, indicating a natural preference for social novelty. In both tests the following parameters were recorded by an observer sitting at 200 cm distance from the box for a 2x5 minute-period: the number of entries into the chamber relative to the total number of entries, and the time of interaction with the stranger relative to the total time of interaction. The number of entries was counted when both the head and the four paws of the tested mouse have entered into the chamber. The time of interaction was measured when the tested mouse was at least at 3 cm from the cage. The floors and the walls of the arena were cleaned with sodium hypochlorite solution between the tests.

3.6. Laboratory tests

3.6.1. Blood alcohol concentration (BAC) assay

The amount of alcohol consumed by mice was calculated by bottle weight each day, and BAC was determined only on the 4th day (immediately after binge drinking) and on the 5^{th} day (24 hours after binge drinking). The mice were decapitated and trunk blood was collected after the behavioral tests. Based on previous experiments, drinking in the dark results in alcohol intakes between 3.5-5.0 g/kg alcohol in mice [58, 59]. The intake of this amount of alcohol should produce a BAC of 0.08 g/dL within 2 hours in C57BL/6 mice [58, 59]. However, in the present experiments mice did not always reach the alcohol level that is characteristic for binge drinking, therefore, mice with BAC lower than 0.08 g/dL were excluded from the statistical analysis. The protocol of alcohol administration in rats was based on a previous study in which 20% alcohol was administered at dose of 3 g/kg [51]. This amount of alcohol produced a BAC of 197.5± 19 mg/dl that was determined from the trunk blood of the rats after decapitation, at 30 min after the IP administration [51].

Ethanol was determined from the plasma obtained by centrifugation of the trunk blood, immediately after sample collection by an enzymatic kit on cobas c502 analyzer (Roche Diagnostics). The sensitivity of the assay was 10.1 mg/dL (0.01 g/dL).

3.6.2. Polymerase chain reaction (PCR)

For the determination of the hypothalamic CRF and AVP expression quantitative reverse transcription polymerase chain reaction (PCR) was performed. First the rats were decapitated, their brains were removed and dissected in a Petri dish filled with ice-cold Krebs solution. The hypothalamus was isolated from each rat according to a stereotaxic atlas of the rat brain [60], after the following coordinates: rostro-caudal (RC) +2.6 - -2.6 mm, medio-lateral (ML) +1,5 - -1,5 mm, dorso-ventral (DV) +7 - +10 mm (**Figure 14**). The tissue samples were stored in 1 mL of TRIzol (Thermo Fisher, USA) in Eppendorf tubes and kept in a freezer at -80°C.

The tissue samples underwent ultrasonic homogenization (Branson Sonifier 250, Emerson, USA), then 200 µL of chloroform was added to each sample. Following 10 minutes of incubation at room temperature, the samples were centrifuged for 15 minutes at 13000 g (Heraeus Fresco 17, Thermo Fisher Scientific, USA). Approximately 500 µL of supernatant was collected from each tube and transferred to new Eppendorf tubes containing 600 µL of 96% alcohol that were stored overnight at -20°C.

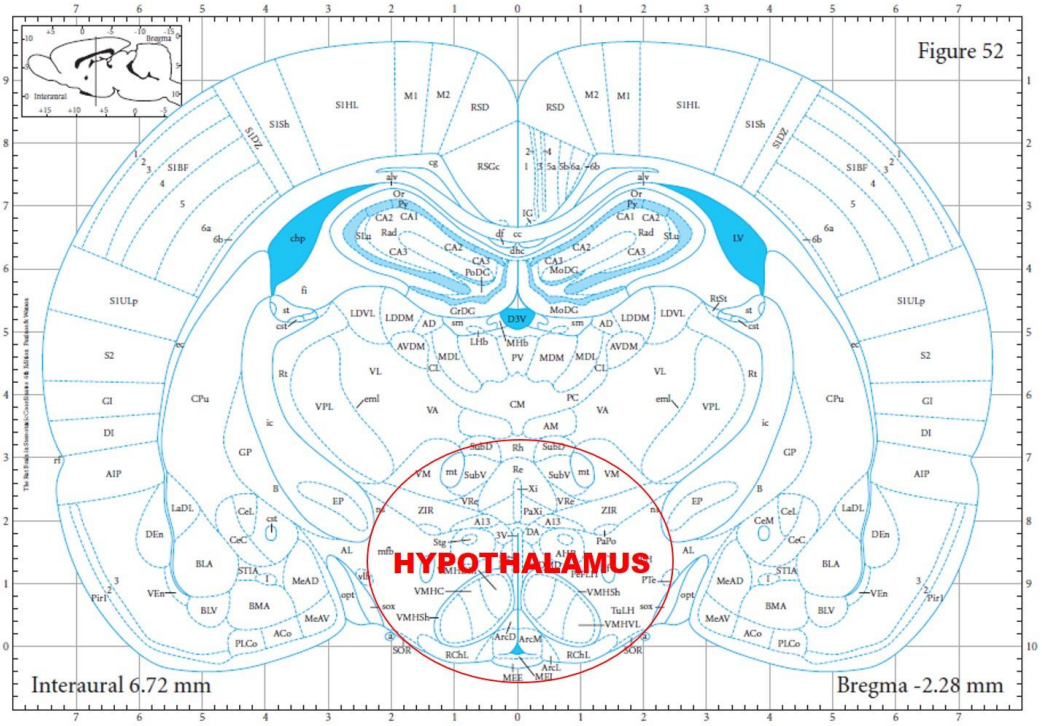


Figure 14. The dissection of the hypothalamus according to a stereotaxic atlas of the rat brain [11].

On the following day, GeneJET RNA Purification Kit (Thermo Fisher Scientific, USA) was used according to the manufacturer's instructions. The concentration of the purified samples was calculated based on the average of three measurements with a spectrophotometer (NanoDrop OneC, Thermo Fisher Scientific, USA). The RNA samples were deemed uncontaminated if the 260/280 nm ratio was between 1.8 and 2.2.A volume containing 300 ng of RNA was obtained from each sample for cDNA synthesis. The first strand cDNA was synthesized using the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions.

The qPCR reaction mix was prepared using the Luminaris Color HiGreen Low ROX qPCR Master Mix (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. A total volume of 10 µL of reaction mix was prepared, containing 5 µL of Master Mix, 0.3 µL of forward primer, 0.3 µL of reverse primer, 1.67 µL of cDNA and 2.73 µL of nuclease-free water. The custom primers corresponding to the CRF and AVP genes are shown in **Table 1**.

Gene	Forward	Reverse
CRF	5′-TGG TGT GGA GAA ACT CAG AGC-3′	5′-CAT GTT AGG GGC GCT CTC TTC-3′
AVP	5′-CTG ACA TGG AGC TGA GAC AGT-3′	5′-CGC AGC TCT CGT CGC T-3′
Gapdh	5′-CGG CCA AAT CTG AGG CAA GA-3′	5′-TTT TGT GAT GCG TGT GTA GCG-3′

Table 1. The custom primers

The mix was placed in a thermal cycler (C1000 Touch Thermal Cycler, BioRad) which was programmed according to the cycling protocol in **Table 2**. The expression of each gene relative to *Gapdh* was determined using the ΔΔCT method.

Phase	Temperature °C	Time	Number of Cycles
UDG pre-treatment	50	2 min	1
Initial denaturation	95	10 min	1
Denaturation	95	15 s	40
Annealing	60	30 s	40
Extension	72	30 s	40

Table 2. The cycling protocol

3.6.3. Enzyme-Linked Immunosorbent Assay (ELISA)

For the determination of the hypothalamic CRF and AVP concentration an *in vitro* homogenization was performed [61, 62] that was followed by sandwich ELISA.

In order to do so, first the rats were decapitated, their brains were removed and dissected in a Petri dish filled with ice-cold Krebs solution. The hypothalamus was isolated from each rat according to the stereotaxic atlas of the rat brain, as previously described [60]. The samples were dissolved in 500 µl acetic acid at 2M concentration in Eppendorf tubes and immersed in boiling water for 5 min. Then, the samples were homogenized with ultrasonic homogeniser (Branson Sonifier 250) on ice for 30 sec. The homogenizates were centrifuged twice at 10000 rpm at 4°C for 20 min after of which the supernatants were separated and liophylisated for further determinations.

To the wells of the plate coated with highly purified antibody against mouse/rat CRF/AVP, standard antigen or sample was added for the 1st step, immunoreaction. After the 1st step incubation and plate washing, biotinylated rabbit anti rat CRF/AVP antibody was added as the 2nd step to form CRF/AVP antibody-antigen-biotinylated CRF/AVP antibody complex on the surface of the wells. After the 2nd step incubation and rinsing out excess biotinylated antibody, horseradish peroxidase labeled streptoavidin was added for binding to biotinylated CRF antibody. Finally, horseradish peroxidase enzyme activity was determined by 3,3',5,5'-tetramethyl benzidine addition and a typical calibration curve was generated. The CRF/AVP concentrations of the extracted samples were corrected for extraction recovery and expressed in ng/ml.

For the determination of the plasma ACTH concentration sandwich ELISA was performed, and the kit that was very similar to the ones used to determine hypothalamic CRF and AVP concentration. To the wells of the plate coated with highly purified antibody against rat/mouse ACTH and, standard antigen or sample was added for the 1st step, immunoreaction. After the 1st step incubation and plate washing, biotinylated rabbit anti rat ACTH antibody was added as the 2nd step to form ACTH antibody-antigen-biotinylated ACTH antibody complex on the surface of the wells. After the 2nd step incubation and rinsing out excess biotinylated antibody, horseradish peroxidase labeled streptoavidin was added for binding to biotinylated ACTH antibody. Finally, horseradish peroxidase enzyme activity was determined by 3,3',5,5'-tetramethyl benzidine addition and a typical calibration curve was generated. The ACTH concentrations of the extracted samples were corrected for extraction recovery and expressed in ng/ml.

3.6.4. Chemofluorescent assay

For the determination of the plasma CORT concentration a chemofluorescent assay was perfomed as described originally by Purves and Sirett, and modified later by Zenker and Bernstein [63, 64]. According to this method, the trunk blood of the rats was collected into heparinized tubes and centrifuged for at 3000 rpm 10 min, then 200 µl aliquots of the medium were transferred to centrifuge tubes. Meanwhile, a reagent blank of 200 µl of distilled water and 2 CORT standards of the same volume containing 25 µg or 50 µg, respectively, were prepared. Five ml of methylene chloride was delivered with an automatic pipette to each tubes and rocked for 30 min to allow complete extraction of CORT by the solvent. The extract was centrifuged for 10 min at 3000 rpm. To eliminate any aqueous phase, approximately 3.2 ml of the lower hydrophobic phase was aspired with a glass syringe then transferred into another centrifuge tube. Four ml of fluorescent reagent (stable mixture of 2.4 volumes of sulfuric acid and 1.0 volume of 50 % v/v aqueous ethyl-alcohol) was added to the extract. The tubes were shaken vigorously for 15 seconds, then allowed to stand at room temperature for 1 hour, in order to permit the maximum fluorescence from CORT. Emission intensity was measured from the lower sulfuric acid layer with Hitachi 204-A fluorescent spectrophotometer at 456 nm extinction and 515 nm emission wave-lengths. The concentration of CORT of the samples was calculated from the values of the standards and expressed as µg/100 ml.

3.6.5. Superfusion

The striatal DA, amygdalar GABA and hippocampal GLU release were measured by means of an *in vitro* superfusion system and a liquid scintillation counter, described originally by Gaddum, and improved later by Harsing and Vizi [65, 66]. The rats were decapitated, their brains were removed and dissected in a Petri dish filled with ice-cold Krebs solution. The striatum, amygdala and hippocampus were isolated from each rat according to a stereotaxic atlas of the rat brain [60], after the following coordinates: RC + 4.0 to -1.0 mm, ML + 1.0 to + 5.0 mm, DV + 3.0 to + 8.0 mm for the striatum (**Figure 15**); RC 0.0 to - 2.0 mm, ML + 3.0 to + 6.0 mm, DV +7.0 to + 10.0 mm for the amygdala (**Figure 16**); and RC - 4.0 to - 6.0 mm, ML + 2.0 to + 5.0 mm, DV + 3.0 to + 8.0 mm for the hippocampus (**Figure 17**). The brain tissue was cut into 3 µm slices with a tissue chopper (McIlwain Inc., USA). and the slices obtained were incubated for 30 min in 8 ml of Krebs solution, submerged in a water bath at 37 °C and gassed through a single-use needle with a mixture of 5% CO2 and 95% O2.

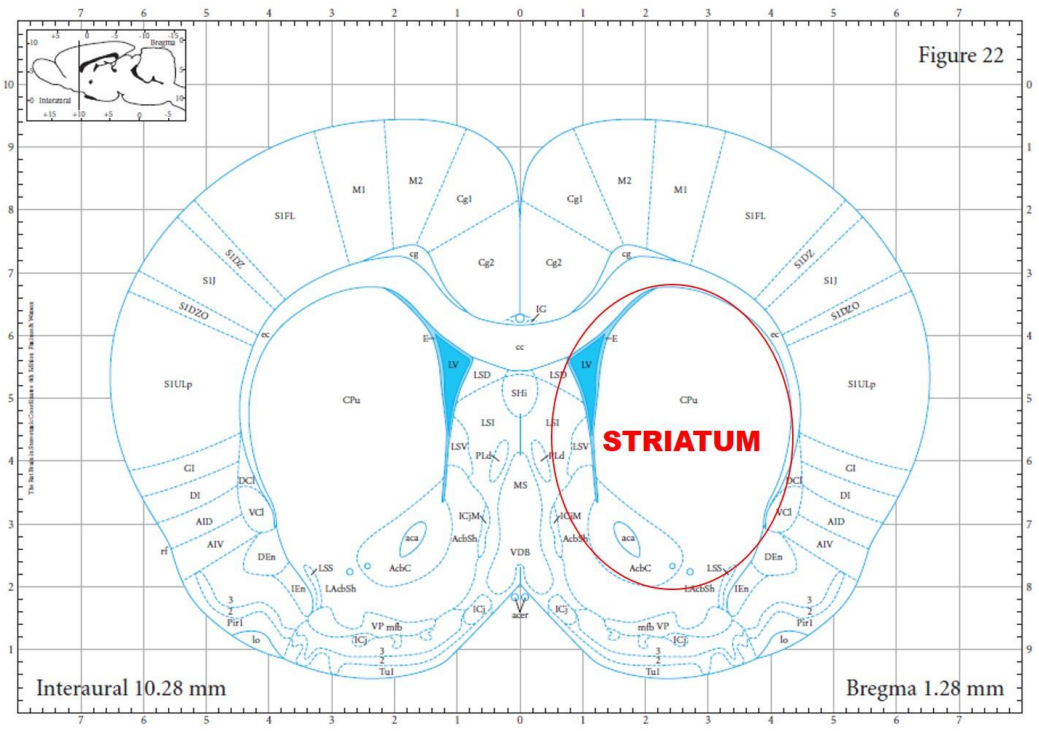


Figure 15. The dissection of the striatum according to a stereotaxic atlas of the rat brain [11].

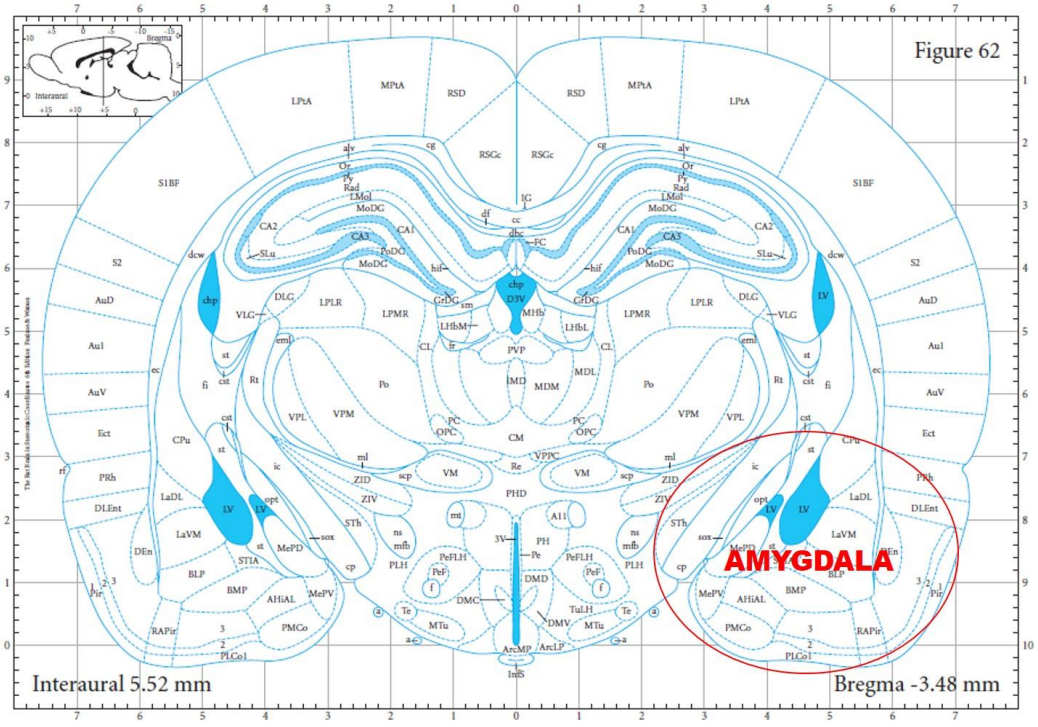


Figure 16. The dissection of the amygdala according to a stereotaxic atlas of the rat brain [11].

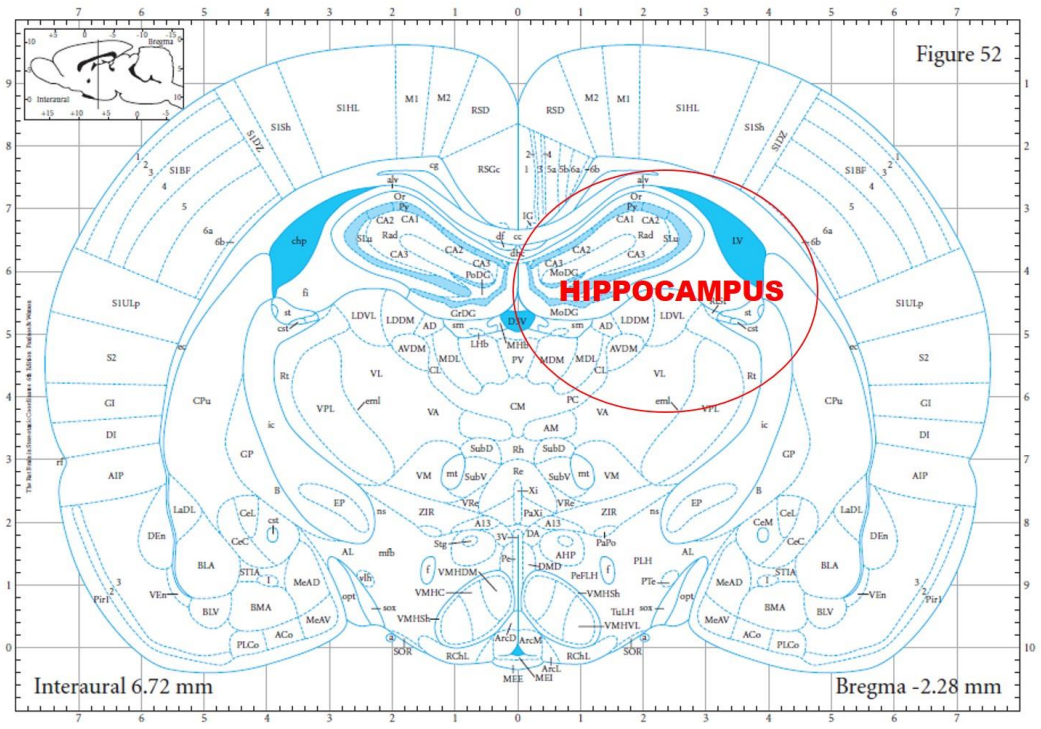


Figure 17. The dissection of the hippocampus according to a stereotaxic atlas of the rat brain [11].

During the incubation, 15 mM of [^{3}H]DA, [^{3}H]GABA or [^{3}H]GLU were added to the incubation medium, depening on the brain slices which were incubated. After incubation, the tritium-labelled brain slices were transferred to each of the four cylindrical perspex chambers of a superfusion system (Experimetria Ltd., Hungary). Golden electrodes were attached to both halves of the chambers and connected to an ST-02 electrical stimulator (Experimetria Ltd., Hungary). A multichannel peristaltic pump (Gilson Minipuls 2) was used to perfuse the brain slices at a constant rate of 300 µl/min. The slices were superfused for 30 min to allow tissue equilibrium, and then the superfusates were collected in Eppendorf tubes by a multichannel fraction collector (Gilson FC 203B). After two minutes, electrical stimulation consisting of square-wave impulses (duration: 2 min, voltage: 100 V, pulse length: 5 ms, frequency: 10 Hz) was delivered to each of the four chambers. The total collecting time was 32 min (4x16 samples, 2 min each) and the peak of the fractional release was observed at 14 minutes. The brain tissue was removed from each chamber and solubilized in 200 ml of Krebs solution, using an ultrasonic homogenizer (Branson Sonifier 250). After the addition of 3 ml of scintillation fluid to the samples and the remaining brain tissue, the radioactivity was measured with a liquid scintillation spectrometer (Tri-carb 2100TR, Packard Inc., USA) and expressed in count per minute (CPM). The fractional release was calculated as the ratio between the radioactivity of the samples and that of the remaining brain tissue.

3.7. Statistical analysis

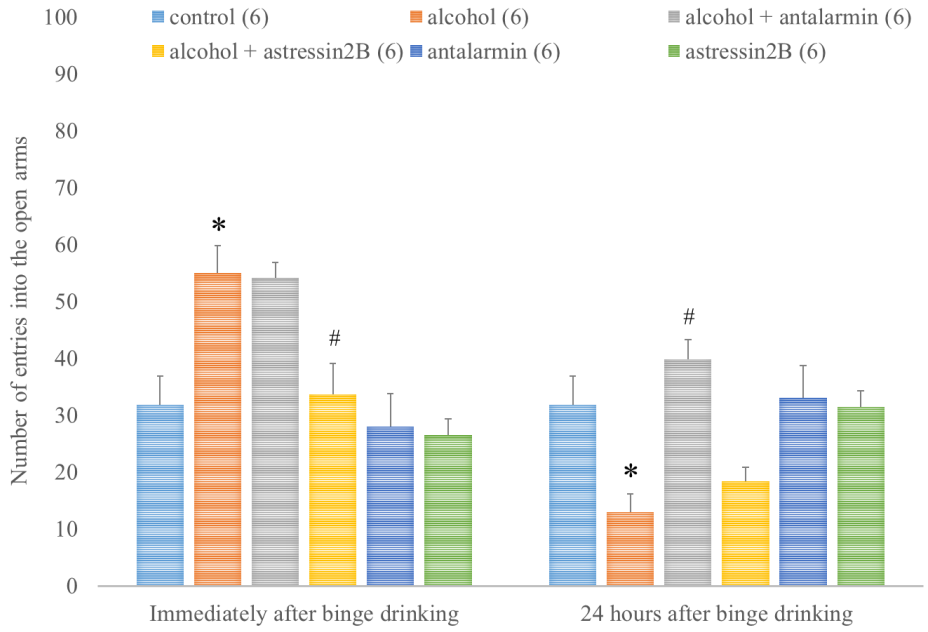
In case of the experiments with mice, statistical analysis of the results was performed by analysis of variance (GraphPad Prism, GraphPad Software Inc., USA). The differences between groups were determined by one-way ANOVA, followed by Tukey's post-hoc test for pair-wise comparisons. In case of the experiments with rats, statistical analysis of the results was performed by ANOVA (SPSS Inc., USA). A two-way 2 (alcohol or saline) x 3 (Antalarmin or Astressin2B or saline) ANOVA was performed with estimated marginal means calculated and followed by Bonferroni post-hoc test for pair-wise comparisons. In both cases, a probability level of less then 0.05 was accepted as indicating a statistically significant difference.

4. RESULTS

On the 4th day (immediately after binge drinking), the number of entries into and the time spent in the open arms of the elevated plus-maze increased significantly in mice exposed to alcohol, compared to the control. Consequently, mice exposed to alcohol spent significantly more time with swimming and climbing, and significantly less time with floating in the water, when compared to the control. These anxiolytic and antidepressant effects of alcohol were decreased significantly by astressin$_2$B, but not the antalarmin (**Figures 18-19**). The number of entries to the stranger was not affected in the social interaction tests, but mice exposed to alcohol spent significantly more time with the stranger in both social interaction tests, when compared to the control. These signs of enhanced sociability and preference for social novelty were reduced significantly by antalarmin, but nost astressin$_2$B (**Figures 20-21**).

On the 5th day (24 hours after binge drinking), the number of entries into and the time spent in the open arms were decreased significantly in mice exposed previously to alcohol, compared to the control. Also, mice exposed previously to alcohol spent significantly less time with swimming and climbing, and significantly more time with floating in the water, when compared to the control. These signs of anxiety and depression were reversed significantly by antalarmin, but not astressin2B (**Figures 18-19**). However, previous exposure to alcohol did not affect the number of entries to or the time spent with the stranger in either of the social interaction tests. Accordingly, neither antalarmin, nor astressin2B did influence considerably the sociability and preference for social novelty of the mice (**Figures 20-21**).

A.



B.

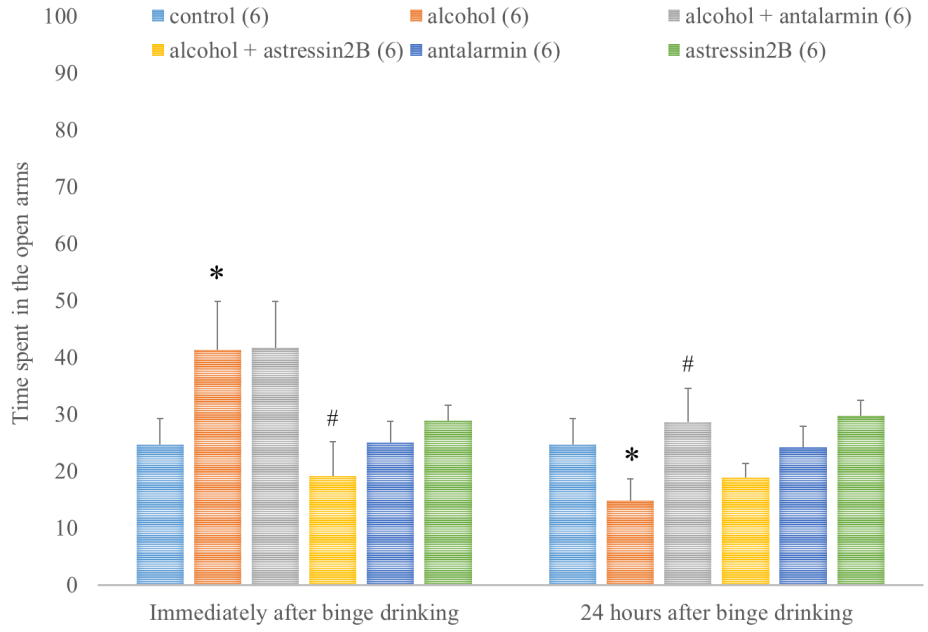
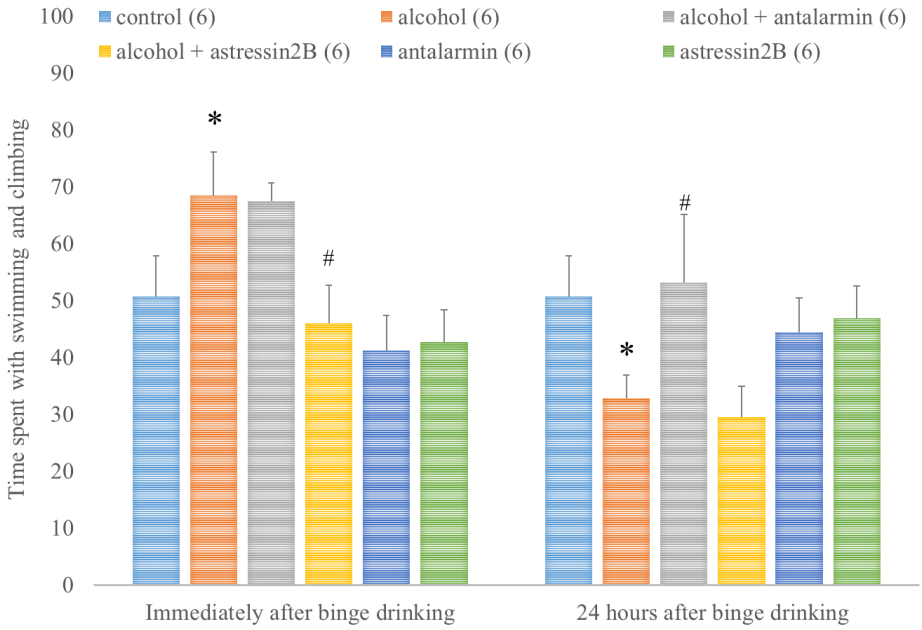


Figure 18. The effects of binge drinking on the number of entries into (A) and the time spent in the open arms (B) in mice investigated in an elevated plus-maze test for signs of anxiety. Values are presented as means ± SEM; statistically significant difference was accepted for $p<0.05$ and indicated with * for alcohol *vs*. control, and # for alcohol + CRF antagonist *vs.* alcohol alone.

A.



B.

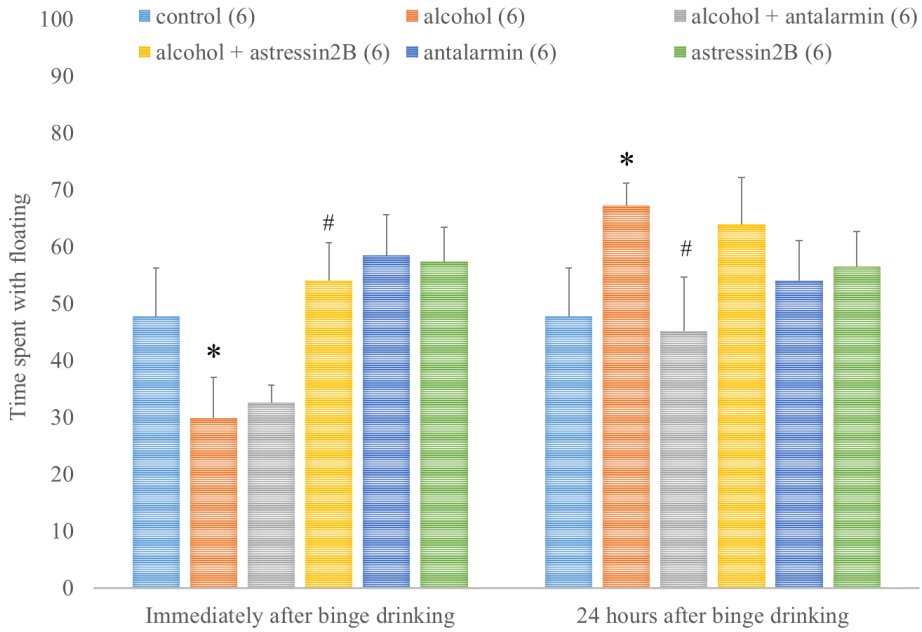
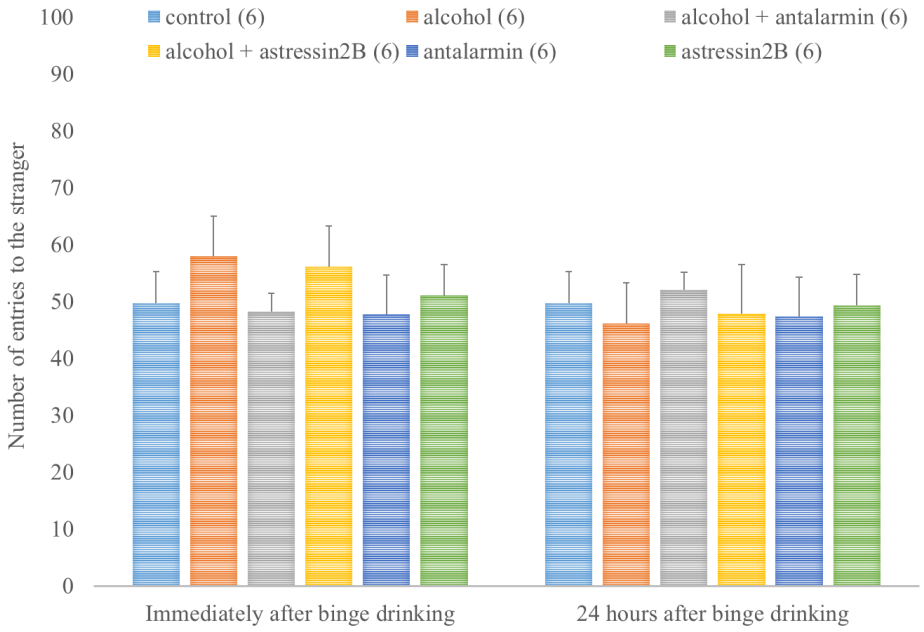


Figure 19. The effects of binge drinking on the time spent with swimming and climbing (A), and floating (B) in mice investigated in a forced swim test for signs of depression. Values are presented as means ± SEM; statistically significant difference was accepted for $p<0.05$ and indicated with * for alcohol *vs.* control, and # for alcohol + CRF antagonist *vs*. alcohol alone.

A.



B.

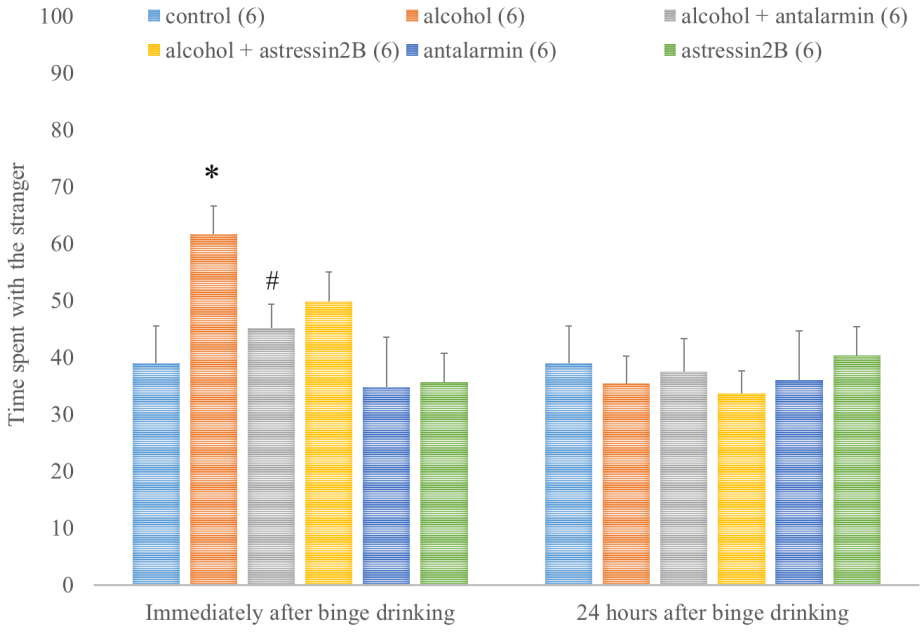
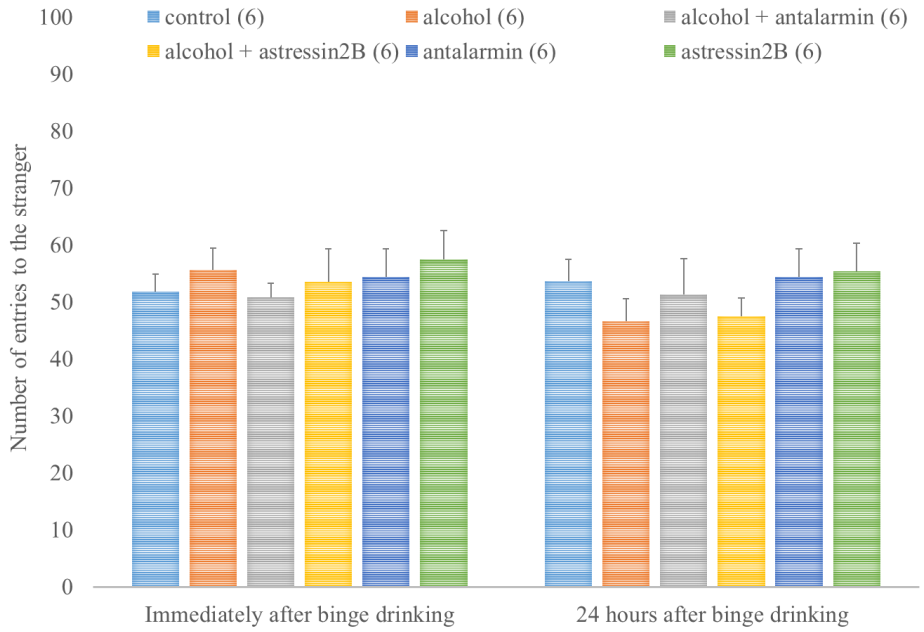


Figure 20. The effects of binge drinking on the number of entries to (A) and the time spent with the stranger (B) in mice investigated in a three-chamber social interaction test for their sociability. Values are presented as means ± SEM; statistically significant difference was accepted for $p<0.05$ and indicated with * for alcohol *vs.* control, and # for alcohol + CRF antagonist *vs.* alcohol alone.

A.



B.

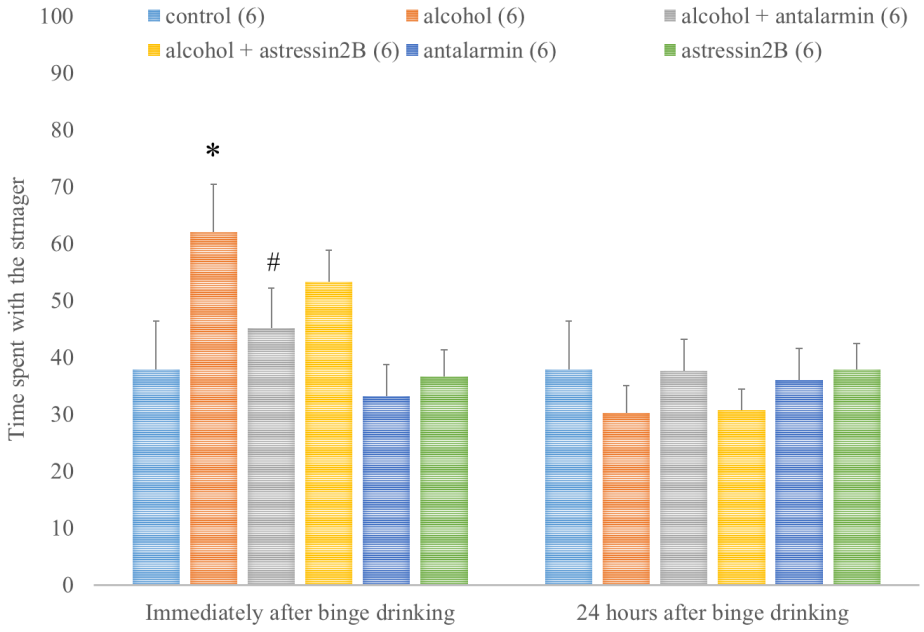


Figure 21. The effects of binge drinking on the number of entries to (A) and the time spent with the stranger (B) in mice investigated in a three-chamber social interaction test for their preference for social novelty. Values are presented as means ± SEM; statistically significant difference was accepted for $p<0.05$ and indicated with * for alcohol *vs.* control, and # for alcohol + CRF antagonist *vs.* alcohol alone.

The statistical analysis was summarized in a separate table for each test (**Tables 3-6**).

Number of entries into the open arms		
Groups	Binge drinking	Hangover
Alcohol vs. Control	F (5, 30) = 9.826 P = 0.0063	F (5, 30) = 6.419 P = 0.0249
Alcohol vs. Alcohol + Antalarmin	F (5, 30) = 9.826 P > 0.999	F (5,30) = 6.419 P = 0.0006
Alcohol vs. Alcohol + Astressin2B	F (5, 30) = 9.826 P = 0.0147	F (5,30) = 6.419 P = 0.9311
Time spent in the open arms		
Groups	Binge drinking	Hangover
Alcohol vs. Control	F (5, 30) = 2.263 P = 0.0423	F (5, 30) = 2.006 P = 0.0440
Alcohol vs. Alcohol + Antalarmin	F (5, 30) = 2.263 P > 0.999	F (5, 30) = 2.006 P = 0.0180
Alcohol vs. Alcohol + Astressin2B	F (5, 30) = 2.2630 P = 0.0149	F (5, 30) = 2.006 P = 0.9804

Table 3. The statistical analysis for the elevated plus-maze test

Time spent with swimming and climbing		
Groups	Binge drinking	Hangover
Alcohol vs. Control	F (5, 30) = 4.295 P = 0.0298	F (5, 30) = 1.798 P = 0.0508
Alcohol vs. Alcohol + Antalarmin	F (5, 30) = 4.295 P > 0.999	F (5, 30) = 1.798 P = 0.0365
Alcohol vs. Alcohol + Astressin2B	F (5, 30) = 4.295 P = 0.0107	F (5, 30) = 1.798 P = 0.994
Time spent with floating		
Groups	Binge drinking	Hangover
Alcohol vs. Control	F (5, 30) = 4.223 P = 0.0335	F (5, 30) = 1.429 P = 0.0486
Alcohol vs. Alcohol + Antalarmin	F (5, 30) = 4.223 P = 0.9996	F (5, 30) = 1.429 P = 0.3547
Alcohol vs. Alcohol + Astressin2B	F (5, 30) = 4.223 P = 0.0870	F (5, 30) = 1.429 P = 0.9996

Table 4. The statistical analysis for the forced swim test

Number of entries to the stranger		
Groups	Binge drinking	Hangover
Alcohol vs. Control	F (5, 30) = 0.5843 P = 0.8988	F (5, 30) = 0.1044 P = 0.9987
Alcohol vs. Alcohol + Antalarmin	F (5, 30) = 0.5843 P = 0.8175	F (5, 30) = 0.1044 P = 0.9863
Alcohol vs. Alcohol + Astressin2B	F (5, 30) = 0.5843 P > 0.999	F (5, 30) = 0.1044 P > 0.999
Time spent with the stranger		
Groups	Binge drinking	Hangover
Alcohol vs. Control	F (5, 30) = 3.782 P = 0.049	F (5, 30) = 0.1693 P > 0.999
Alcohol vs. Alcohol + Antalarmin	F (5, 30) = 3.782 P = 0.0261	F (5, 30) = 0.1693 P = 0.998
Alcohol vs. Alcohol + Astressin2B	F (5, 30) = 3.782 P = 0.6125	F (5, 30) = 0.1693 P > 0.999

Table 5. The statistical analysis for the sociability test

Number of entries to the stranger		
Groups	Binge drinking	Hangover
Alcohol vs. Control	F (5, 30) = 0.2391 P = 0.9940	F (5, 30) = 0.3691 P = 0.9255
Alcohol vs. Alcohol + Antalarmin	F (5, 30) = 0.2391 P = 0.9827	F (5, 30) = 0.3691 P = 0.9866
Alcohol vs. Alcohol + Astressin2B	F (5, 30) = 0.2391 P = 0.9996	F (5, 30) = 0.3691 P = 0.9866
Time spent with the stranger		
Groups	Binge drinking	Hangover
Alcohol vs. Control	F (5, 30) = 2.779 P = 0.0138	F (5, 30) = 0.4130 P > 0.999
Alcohol vs. Alcohol + Antalarmin	F (5, 30) = 2.779 P = 0.0490	F (5, 30) = 0.4130 P = 0.9399
Alcohol vs. Alcohol + Astressin2B	F (5, 30) = 2.779 P = 0.9387	F (5, 30) = 0.4130 P > 0.999

Table 6. The statistical analysis for the preference for social novelty test

Hypothalamic CRF mRNA expression of rats was increased by alcohol intoxication and withdrawal, and these stimulatory effects were reduced by antalarmin, but not astressin2B (**Figure 22**). On the 5th day, a significant main effect in the alcohol treated group [$F(1,17) = 3.568$, $p < 0.001$] and in the antalarmin-treated group [$F(2,11) = 1.875$, $p < 0.001$], and a significant interaction between the two factors [$F(2,17) = 2.02$, $p < 0.001$] were observed. On the 6th day, a significant main effect in the alcohol treated group [$F(1,17) = 12.476$, $p < 0.001$] and in the antalarmin-treated group [$F(2,11) = 8.535$, $p < 0.001$], and a significant interaction between the two factors [$F(2,17) = 17.435$, $p < 0.001$] were detected. In addition, hypothalamic CRF concentration was increased by alcohol intoxica-tion and withdrawal, and these stimulatory effects were reversed by the selective CRF1, but not CRF2 antagonist (**Figure 23**). On the 5th day, a significant main effect in the alcohol-treated group [$F(1,134) = 39.173$, $p < 0.001$], but no significant main effect in the antagonist-treated group [$F(2,34) = 1.561$, $p = 0.227$], yet a significant interaction between the two factors [$F(1,17) = 3.356$, $p < 0.001$] were observed. On the 6th day, a significant main effect in the alcohol-treated group [$F(1,35) = 10.021$, $p = 0.004$], and no significant main effect in the antagonist-treated group [$F(2,35) = 2.371$, $p = 0.11$], and no significant interaction between the two factors [$F(2,35) = 2.833$, $p = 0.075$] were detected.

Hypothalamic AVP mRNA expression of rats was decreased by alcohol intoxication and withdrawal, but these inhibitory effects were not influenced significantly either by an-talarmin or astressin2B (**Figure 24**). On the 5th day, a significant main effect in the alco-hol-treated group [$F(1,17) = 3.451$, $p < 0.001$], no significant main effect in the antago-nist-treated groups [$F(1,11) = 0.287$, $p = 0.621$], and a significant interaction between the two factors [$F(2,11) = 6.299$, $p < 0.001$] were assessed. On the 6th day, a significant main effect in the alcohol-treated group [$F(1,17) = 6.425$, $p < 0.001$], and a significant interaction between the two factors [$F(2,11) = 10.245$, $p < 0.001$] were noticed. In contrast, hypothalamic AVP concentration was increased by alcohol intoxication and withdrawal, but these stimulatory effects were not affected by none of the se-lective CRF antagonists (**Figure 25**). On the 5th day, a significant main effect in the al-cohol-treated group [$F(1,35) = 85.4$, $p < 0.001$], but no significant main effect in the an-tagonist-treated groups [$F(2,35) = 0.562$, $p = 0.576$], and no significant interaction be-tween the two factors [$F(2,35) = 0.022$, $p = 0.978$] were assessed. On the 6th day, a sig-nificant main effect in the alcohol-treated group [$F(1,35) = 80.680$, $p < 0.001$], but no significant main effect in the antagonist-treated groups [$F(2,35) = 0.022$, $p = 0.978$], and no significant interaction between the two factors [$F(2,35) = 0.189$, $p = 0.829$] were noticed.

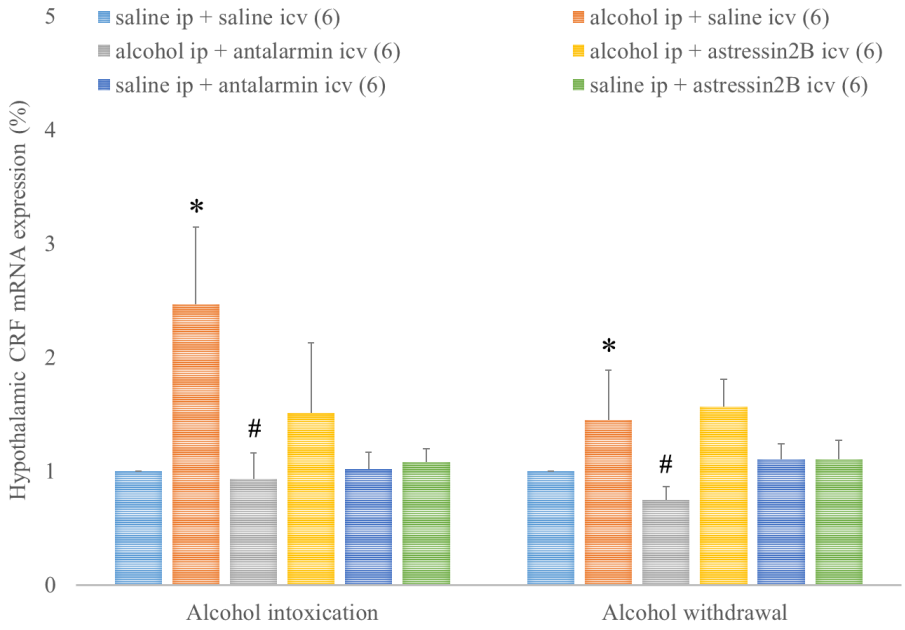


Figure 22. The effects of alcohol intoxication and withdrawal on hypothalamic corticotropin-releasing factor (CRF) expression in rats and the impacts of antalarmin and astressin2B on these effects. Values are presented as means ± SEM; statistically significant difference was accepted for $p<0.05$ and indicated with * for alcohol ip + saline icv *vs.* saline ip + saline icv and with # for alcohol ip + antalarmin icv *vs.* alcohol ip + saline icv.

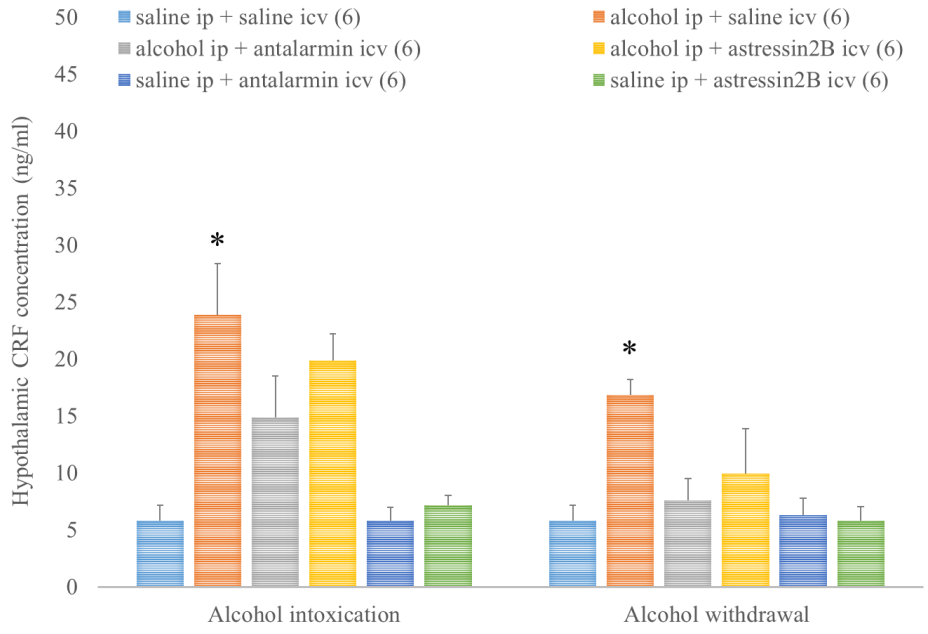


Figure 23. The effects of alcohol intoxication and withdrawal on hypothalamic corticotropin-releasing factor (CRF) concentration in rats and the impacts of antalarmin and astressin2B on these effects. Values are presented as means ± SEM; statistically significant difference was accepted for $p<0.05$ and indicated with * for alcohol ip + saline icv *vs.* saline ip + saline icv and with # for alcohol ip + antalarmin icv *vs.* alcohol ip + saline icv.

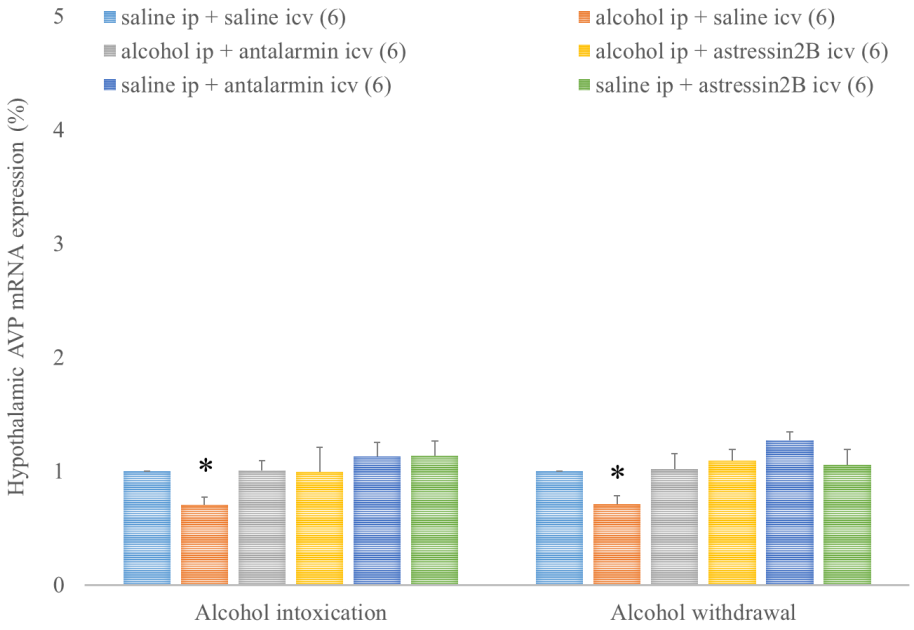


Figure 24. The effects of alcohol intoxication and withdrawal on the hypothalamic arginine vaso-pressin (AVP) expression in rats and the impacts of antalarmin and astressin2B on these effects. Values are presented as means ± SEM; statistically significant difference was accepted for $p<0.05$ and indicated with * for alcohol ip + saline icv *vs*. saline ip + saline icv and with # for alcohol ip + antalarmin icv *vs*. alcohol ip + saline icv.

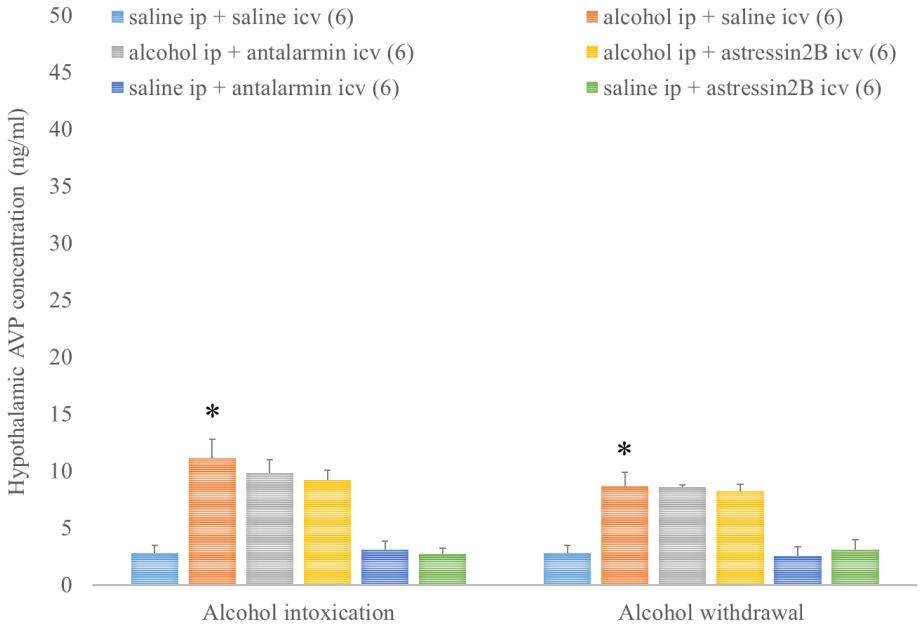


Figure 25. The effects of alcohol intoxication and withdrawal on hypothalamic arginine vasopressin (AVP) concentration in rats and the impacts of antalarmin and astressin2B on these effects. Values are presented as means ± SEM; statistically significant difference was accepted for $p<0.05$ and in-dicated with * for alcohol ip + saline icv *vs*. saline ip + saline icv and with # for alcohol ip + an-talarmin icv *vs*. alcohol ip + saline icv.

Plasma ACTH level of rats was elevated by alcohol intoxication and withdrawal, and these elevations were ameliorated by antalarmin, but not astressin2B (**Figure 26**). On the 5th day, a significant main effect in the alcohol-treated group [$F(1,35) = 64.352$, $p < 0.001$] and a significant main effect in the antagonist-treated group [$F(2,35) = 12.523$, $p < 0.001$], and a significant interaction between the two factors [$F(2,35) = 9.311$, $p < 0.001$] were shown. On the 6th day, a significant main effect in the alcohol-treated group [$F(1,35) = 49.394$, $p < 0.001$] but no significant main effects in the antago-nist-treated group [$F(2,35) = 7.031$, $p = 0.003$], and no significant interaction between the two factors [$F(2,35) = 5.766$, $p = 0.008$] were proved.

In parallel, plasma CORT level of rats was augmented by alcohol intoxication and with-drawal, and these augmentations were attenuated by the selective CRF1, but not CRF2 antagonist (**Figure 27**). On the 5th day, a significant main effect in the alcohol-treated group [$F(1,35) = 30.996$, $p < 0.001$] and a significant main effect in the antago-nist-treated group [$F(2,35) = 3.892$, $p < 0.001$], and a significant interaction between the two factors [$F(2,35) = 4.778$, $p < 0.001$] were shown. On the 6th day, a significant main effect in the alcohol-treated group [$F(1,35) = 62.363$, $p < 0.001$] and a significant main effect in the antagonist-treated group [$F(2,35) = 9.192$, $p < 0.001$], and a significant in-teraction between the two factors [$F(2,35) = 9.765$, $p < 0.001$] were proved.

Alcohol intoxication increased, whereas alcohol withdrawal decreased the striatal DA release, and both effects were reduced by antalarmin, but not astressin2B (**Figure 28**). On the 5th day, a significant main effect in the alcohol-treated group [$F(1,17) = 271.724$, $p < 0.001$], in the antalarmin-treated group [$F(2,11) = 98.352$, $p < 0.001$] and a significant interaction between the two factors [$F(2,11) = 100.708$, $p < 0.001$] were ob-served. On the 6th day, a significant main effect in the alcohol-treated group [$F(1,17) = 319.151$, $p < 0.001$], in the antalarmin-treated group [$F(2,11) = 52.758$, $p < 0.001$] and a significant interaction between the two factors [$F(2,11) = 103.865$, $p < 0.001$] were detected.

Similarly, alcohol intoxication increased, whereas alcohol withdrawal decreased the amygdalar GABA release and both effects were reversed by the selective CRF1, but not CRF2 antagonist (**Figure 29**). On the 5th day, a significant main effect in the alco-hol-treated group [$F(1,17) = 226.989$, $p < 0.001$] and in the antalarmin-treated group [$F(2,11) = 68.927$, $p < 0.001$], and a significant interaction between the two factors [$F(2,11) = 57.735$, $p < 0.001$] were assessed. On the 6th day, a significant main effect in the alcohol-treated group [$F(1,17) = 123.070$, $p < 0.001$], and in the antalarmin-treated group [$F(2,11) = 36.792$, $p < 0.001$] and a significant interaction between the two factors [$F(2,11) = 60.402$, $p < 0.001$] were noticed.

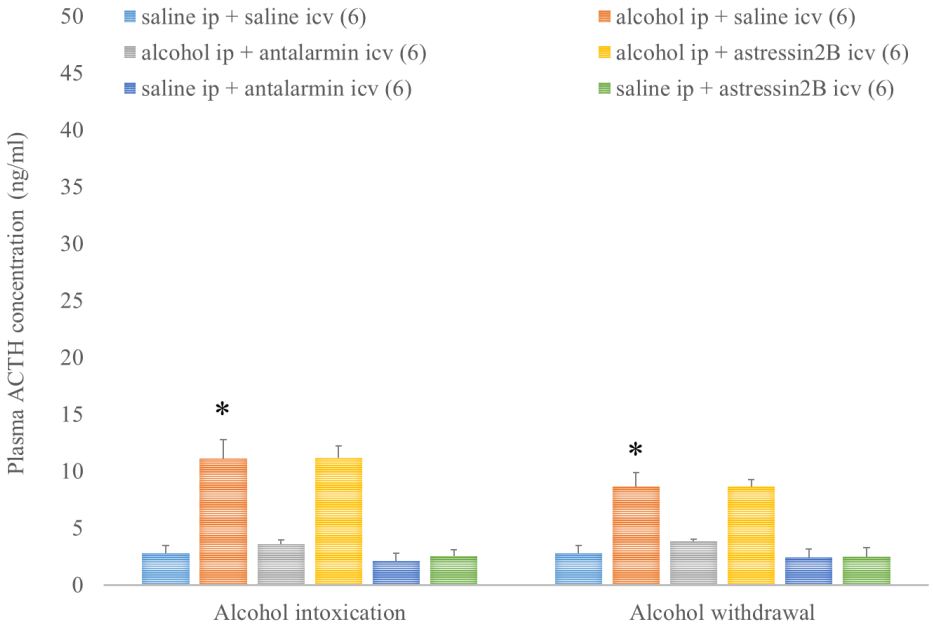


Figure 26. The effects of alcohol intoxication and withdrawal on plasma adrenocorticoptropic hormone (ACTH) concentration in rats and the impacts of antalarmin and astressin2B on these effects. Values are presented as means ± SEM; statistically significant difference was accepted for $p<0.05$ and indicated with * for alcohol ip + saline icv *vs.* saline ip + saline icv and with # for alcohol ip + antalarmin icv *vs.* alcohol ip + saline icv.

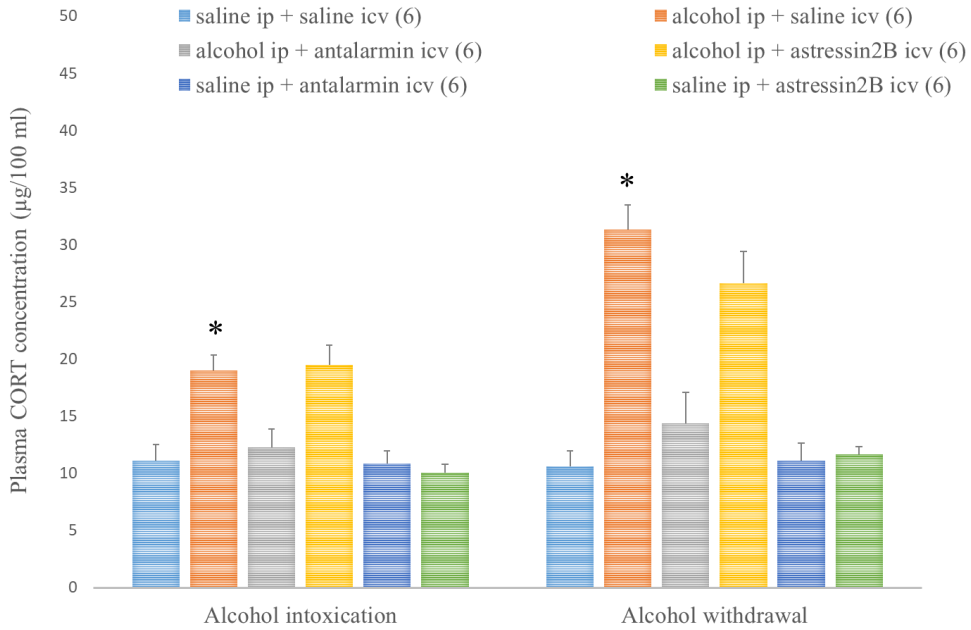


Figure 27. The effects of alcohol intoxication and withdrawal on plasma corticosterone (CORT) concentration in rats and the impacts of antalarmin and astressin2B on these effects. Values are presented as means ± SEM; statistically significant difference was accepted for $p<0.05$ and indicated with * for alcohol ip + saline icv *vs.* saline ip + saline icv and with # for alcohol ip + antalarmin icv *vs.* alcohol ip + saline icv.

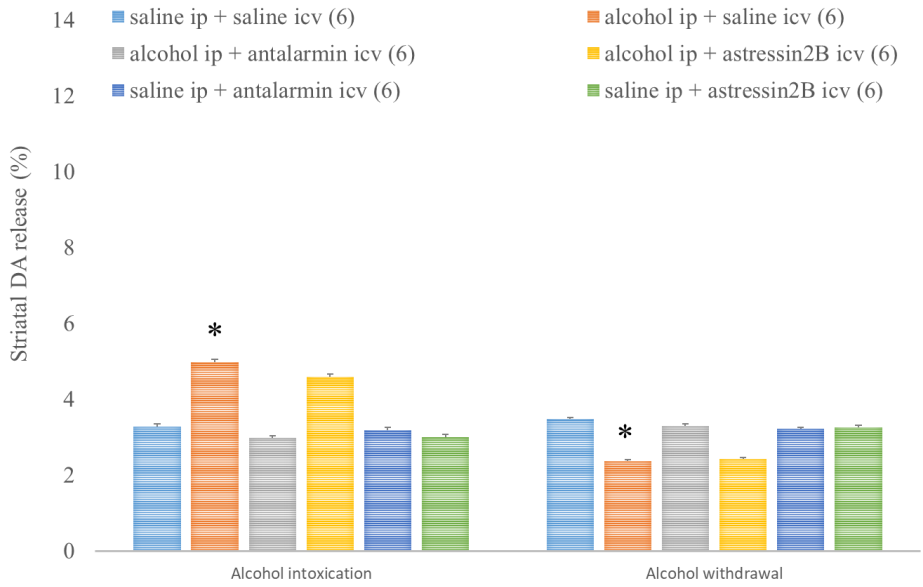


Figure 28. The effects of alcohol intoxication and withdrawal on striatal dopamine (DA) release in rats and the impacts of antalarmin and astressin2B on these effects. Values are presented as means ± SEM; statistically significant difference was accepted for $p<0.05$ and indicated with * for alcohol ip + saline icv *vs*. saline ip + saline icv and with # for alcohol ip + antalarmin icv *vs*. alcohol ip + saline icv.

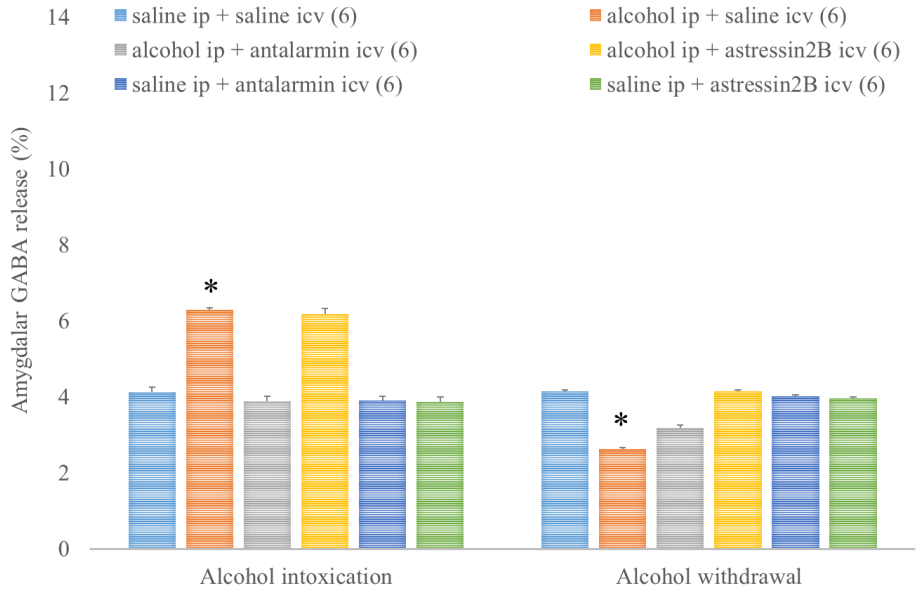


Figure 29. The effects of alcohol intoxication and withdrawal on amygdalar gamma aminobutyric acid (GABA) release in rats and the impacts of antalarmin and astressin2B on these effects. Values are presented as means ± SEM; statistically significant difference was accepted for $p<0.05$ and in-dicated with * for alcohol ip + saline icv *vs*. saline ip + saline icv and with # for alcohol ip + an-talarmin icv *vs*. alcohol ip + saline icv.

Consequently, the hippocampal GLU release was decreased and increased in alcohol intoxication and alcohol withdrawal, respectively, and both effects were antagonized by antalarmin, but not astressin2B (**Figure 30**). On the 5th day, a significant main effect in the alcohol-treated group [$F(1,17) = 663.538$, $p < 0.001$] and in the antalarmin-treated group [$F(2,11) = 172.546$, $p < 0.001$], and a significant interaction between the two factors [$F(2,11) = 257.706$, $p < 0.001$] were shown. On the 6th day, a significant main effect in the alcohol-treated group [$F(1,17) = 305.855$, $p < 0.001$], and in the antalarmin-treated group [$F(2,11) = 97.827$, $p < 0.001$] and a significant interaction between the two factors [$F(2,11) = 82.538$, $p < 0.001$] were proved.

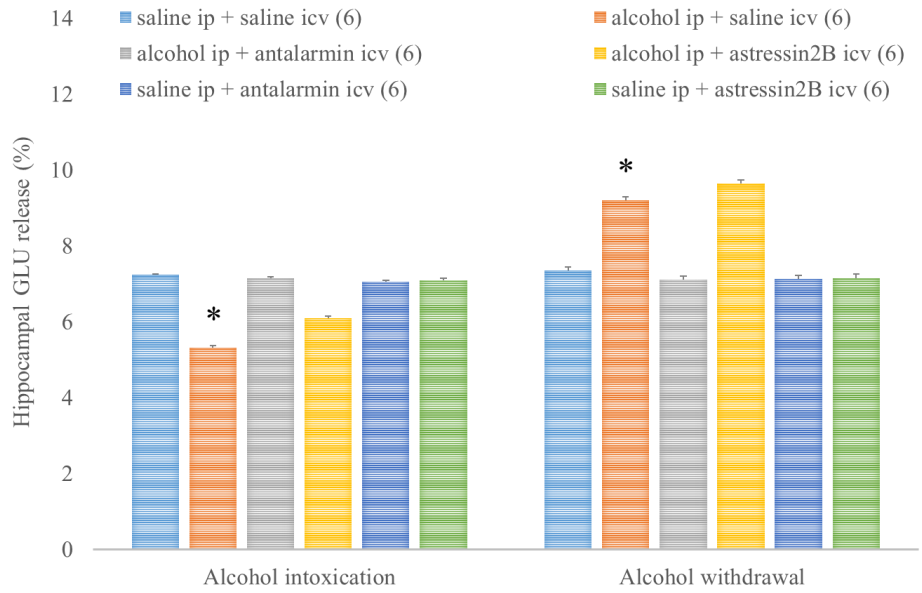


Figure 30. The effects of alcohol intoxication and withdrawal on hippocampal glutamate (GLU) release in rats and the impacts of antalarmin and astressin2B on these effects. Values are presented as means ± SEM; statistically significant difference was accepted for $p<0.05$ and indicated with * for alcohol ip + saline icv *vs*. saline ip + saline icv and with # for alcohol ip + antalarmin icv *vs*. alcohol ip + saline icv.

5. DISCUSSION

5.1. The effects of alcohol intoxication and withdrawal

Our results demonstrate that binge drinking and hangover exert different effects on anxiety-like, depression-like and social behavior.

Binge drinking produces anxiolytic and antidepressant effects when mice are tested immediately after drinking in the dark. Previous studies already suggested that a single cycle of binge drinking is not necessarily associated with anxiety and depression [67, 68]. In concordance, a recent study using a slightly modified version of the drinking in the dark paradigm showed that binge drinking has no short-term effect on the behavior of adolescent C57BL/6 mice, but evokes anxiety- and depressive-like behavior during adulthood [69]. Based on the present study, a single session of binge drinking in adolescent C57BL/6 mice seems to have rather anxiolytic and antidepressant effects. In addition, binge drinking enhances the sociability and the preference for social novelty of male mice, when they are tested immediately after drinking in the dark, a finding that can be related to the anxiolytic and antidepressant effects observed. In general, alcohol is known to have a biphasic effect on social behavior, as low doses increase and high doses decrease the number of social contacts [70, 71]. Furthermore, mice exposed to alcohol spend more time in interaction with a conspecific than the object, regardless of the dose of alcohol [70, 71].

In contrast, mice exposed to alcohol presented anxiety-like and depression-like signs 24 hours after binge drinking, that may correspond for hangover in humans. Hangover is a state that occurs after a single episode of heavy drinking when BAC approaches zero and is associated with a combination of physical signs and affective symptoms, including anxiety and depression. The affective symptoms of hangover - a term used by some authors interchangeably with acute alcohol withdrawal [72, 73] - usually emerge at 10 hours and may persist even after 24 hours following alcohol administration [74, 75]. Indeed, a previous study reported that a history of 30-days of binge drinking elicits negative affect in mice, most notably anxiety-like signs, which emerge after 24 hours of withdrawal and persist for at least 21 days following the last episode of binge drinking [76, 77]. However, in another study that was previously published only a weak negative affect, including a few signs of anxiety-like and depression-like behavior, and no elevation of the plasma CORT levels, were detected after 24 hours of binge drinking in mice [78]. In this study male and female, adolescent and adult mice were subjected to 14 consecutive days of binge drinking using a multi-bottle choice drinking in the dark procedure [78]. The authors of this study concluded that incubation of negative affect during alcohol withdrawal is age-dependent, and not sex-

selective, but also admitted that procedural differences might have accounted for the relatively weak effect of binge drinking upon anxiety-like and depressive-like behavior, when compared to other studies [78]. In addition, based on the present results, binge drinking does not affect the social interaction of male mice, when they are tested 24 hours after drinking in the dark. A recent study already reported that binge drinking has no impact on the sociability and the preference for social novelty of mice, at least when they are tested 24 hours after drinking in the dark [69]. Another study recently published underlined the anxiogenic and cognitive impairing effects of binge drinking [79]. In this study C57BL/6 mice that were exposed to drinking in the dark for a 1-month period and investigated in a battery of behavioral tests, including elevated plus-maze, forced swim and Morris water-maze tests [79]. The authors reached the following conclusions: both biological sex and the age of drinking onset are subject factors that impact voluntary alcohol consumption by mice into old age; binge drinking during later life elicits a negative affective state that is relatively sex-independent; binge drinking during both mature adulthood and old age impairs spatial learning and memory; binge drinking during mature adulthood accelerates deficits in working memory; and mature adult females tend to exhibit more alcohol-induced cognitive impairments than males [79].

Lately, there are several studies investigating the negative affect induced by binge or heavy drinking of alcohol [80-83]. We believe that any inconsistencies found between our study and others investigating the anxiety-like, depression-like and social behavior using the same animal model of binge drinking could be due to the changes in the drinking in the dark paradigm. In our experiments C57BL/6 mice were exposed to alcohol for 4 days, according to the classical drinking in the dark paradigm, even if the animals did not always reach the BAC of 0.08 g/dL within 2 hours. In comparison, in other studies the mice were exposed repeatedly to alcohol, at different times and for longer periods in order to reach the alcohol level that is characteristic for binge drinking [69, 76, 77]. As regards the robust negative affect that was observed 24 hours after a single session of binge drinking in our case, and that was described after several cycles of binge drinking and withdrawal in other cases, we presume that these may also arise from the different methodology. In our experiments, mice with BAC lower than 0.08 g/dL were excluded from the statistical analysis that led to relatively small sample size for each group. It’s also important to mention, that in other experiments no surgical procedures were used before the behavioral tests and mice were not selected based on their alcohol level, therefore a larger sample size and consequently a more complex statistical approach was used that may lead to statistically different outcomes [69, 76, 77].

Our results demonstrate that during alcohol intoxication the activation of the HPA axis is initiated by hypothalamic CRF and reflected by the elevation of plasma CORT and ACTH levels. Besides the stress axis, the reward system is also activated, resulting mainly in increased striatal DA, but also increased amygdalar GABA and decreased hippocampal GLU release. Our results also demonstrate that during alcohol withdrawal, the HPA axis remains activated, but this time the activation is accompanied by the decrease of the striatal DA and amygdalar GABA release, and increase of hippocampal GLU release, which are mediated probably by extrahypothalamic CRF.

Previous studies indicated that alcohol binge/intoxication and withdrawal/negative affect are associated with certain changes of as striatal DA, amygdalar GABA and hippocampal GLU [36, 37]. Acute alcohol administration stimulates the striatal DA release inducing senzation of reward, whereas chronic alcohol administration leads to decrease of the DA release in the striatum, that is manifested in a reward deficit during alcohol withdrawal [84, 85]. This reward deficit can be explained by an increase in the reward threshold caused by the down-regulation of pre-synaptic DA receptors, and a decrease in extracellular DA release caused by the depletion of striatal DA stores that are demasked during alcohol withdrawal [86]. The amygdalar GABA is presumed to play role in the positive, anxiolytic effects of alcohol [87-90]. Acute alcohol consumption facilitates GABA-ergic neurotransmission in CEA via both pre- and post-synaptic mechanisms, whereas chronic alcohol consumption increases baseline GABA-ergic neurotransmission, but not the stimulated GABA release [91]. The hippocampal GLU is believed to play role in the negative, anxiogenic effects of alcohol and the development of aggression observed especially during alcohol withdrawal [51, 92-94]. In general, acute alcohol consumption decreases glutamatergic neurotransmission by down-regulation of GLU receptors, whereas chronic alcohol consumption increases glutamatergic neurotransmission by up-regulation of the GLU receptors and stimulation of GLU release, that might be further enhanced by repeated periods of alcohol withdrawal [91].

5.2. The role of CRF and CRF receptors

Our results indicate that the anxiolytic and antidepressant effects produced by binge drinking are mediated by CRF2, whereas the anxiety-like and depression-like signs induced by hangover are promoted by CRF1. Our results are in concert with the original hypothesis, according to which CRF1 and CRF2 play dualistic roles in the brain [2, 95], with CRF1 promoting activation of the HPA axis, anxiety and depression, and CRF2 mediating anxiolytic

and antidepressant effects. However, the most recent hypothesis states that the role of CRF1 and CRF2 in anxiety and depression is not a matter of simple dualism, but depends on the brain regions and neuronpopulations being activated [7, 8].

A previous study reported that pretreatment with CRF1 antagonist or CRF2 agonist prior to alcohol self-administration could reduce the amount of alcohol administered [96]. The present study using the same animal model suggests that pre-treatment with a selective CRF1 antagonist and a selective CRF2 antagonist could attenuate both the positive, rewarding effects, and the negative, aversive effects of alcohol and alcohol withdrawal, respectively. In this order of thoughts, co-administration of these drugs might prevent spiraling of repeating cycles of binge drinking into alcohol addiction. In addition, selective CRF2 agonists, such as UCN2 and UCN3, may also prove useful in the therapy of alcohol withdrawal, since these ameliorated the anxiety- and depression-like behavior and the activation of the HPA axis developed during acute nicotine withdrawal [53].

Previous studies provided a solid base of evidence to support the role of CRF in the alcohol-induced activation of the HPA axis [97]. First, administration of a CRF antiserum or a CRF antagonist inhibited the stimulatory effect of alcohol on ACTH secretion in rats [98-100]. Second, bilateral destruction of the paraventricular CRF-secreting neurons ameliorated, although did not abolish, the alcohol-stimulated ACTH secretion [101, 102]. Third, administration of alcohol increased the expression of CRF heteronuclear RNA and mRNA levels, and expression of c-Fos mRNA and the Fos protein in the PVN [101, 103, 104]. The present study underlines the role of hypothalamic CRF in the activation of the HPA axis during alcohol intoxication and withdrawal. This finding is supported by the observation that both the expression and concentration of the hypothalamic CRF increased in parallel with the levels of plasma ACTH and CORT, immediately and 24 hours after the last alcohol administration.

Previous studies provided several line of evidence to suggest the role of AVP, as well [97]. First, administration of an AVP antiserum or an AVP antagonist inhibited the stimulatory effect of alcohol on ACTH secretion in rats [100, 105]. Second, removal of endogenous AVP in rats previously exposed to bilateral destruction of the paraventricular neurons diminished partially the alcohol-stimulated ACTH secretion [101]. Third, administration of alcohol increased the expression of AVP heteronuclear RNA and mRNA levels [103]. However, the present study questions the role of the hypothalamic AVP in the activation of the HPA axis during alcohol intoxication and withdrawal. This finding is based on the speculation that the decreased hypothalamic AVP expression and increased

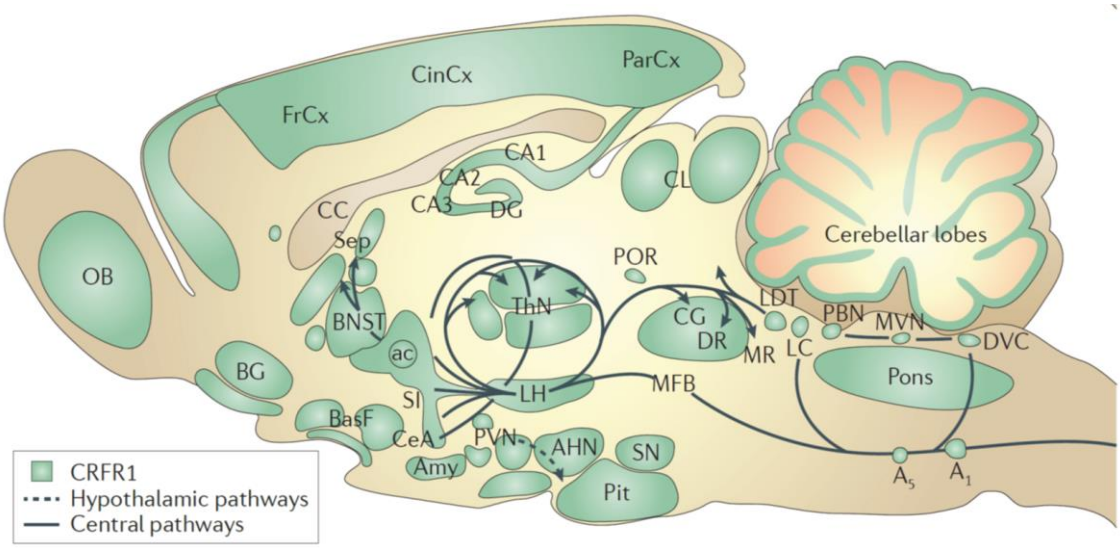
hypothalamic AVP concentration immediately and 24 hours after the last alcohol administration represents rather a decreased release than an increased synthesis of hypothalamic AVP, a process that might have resulted from the negative feedback of plasma glucocorticoids and ACTH or could be related to another function of AVP, such as water retention.

As for the role of CRF1 in the alcohol-induced activation of the HPA axis, previous *in vivo* experiments already indicated that alcohol-induced up-regulation of the paraventricular CRF1 expression [106], and the alcohol-induced ACTH secretion could be blocked by non-selective CRF receptor antagonist astressin and selective CRF1 antagonist NBI 30775 [107]. In contrast, alcohol administration was unable to produce up-regulation of CRF2 expression in the PVN [106], and the alcohol-induced ACTH secretion could not be blocked by selective CRF2 antagonist astressin2B [107].

Also, the role if CRF1 in the changes of the striatal DA, amygdalar GABA and hippocampal GLU was suggested by our previous *in vivo* experiments, according to which the changes in striatal dopamine release and locomotor activity observed during acute withdrawal following chronic nicotine administration are mediated by CRF1, but not CRF2, receptors [52]. Moreover, our previous *in vitro* experiments indicated that the striatal DA and amygdalar GABA release can be stimulated by non-selective CRF1 agonists and this stimulatory effect can be reversed by a selective CRF1 antagonist, but not a selective CRF2 antagonist [108-110]. Nevertheless, they also suggested the existence of two apparently opposing CRF systems, through which CRF and the urocortins might modulate GLU and acetylcholine release in the hippocampus [110, 111].

Overall, the present study emphasizes the role of CRF and CRF1 in the changes of hypothalamic neurohormones and extrahypothalamic neurotransmitters observed during alcohol intoxication and withdrawal, and excludes the role of CRF receptors in the alcohol-induced alteration of the hypothalamic AVP (**Figure 31**). However, future studies using modern techniques of CRF overexpression and global or local CRF1 and CRF2 knockout animal models or targeting specific brain regions and neuron populations with CRF receptor agonists and antagonists are yet to determine the intimate brain regions and pathways involved in alcohol intoxication and withdrawal (**Figure 32**).

A.



B.

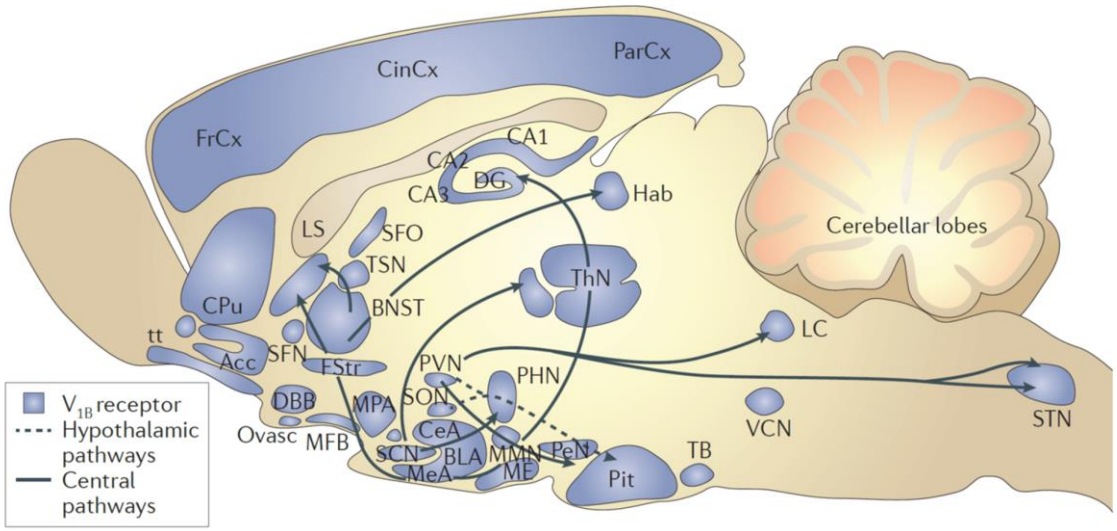
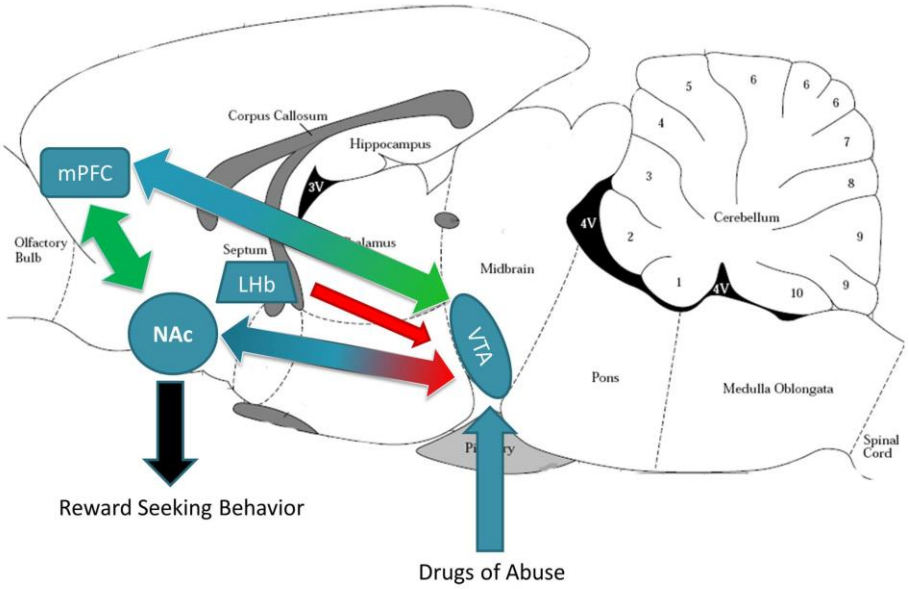


Figure 31. The possible neuropeptides and receptors involved in alcohol intoxication and withdrawal [44].



B.

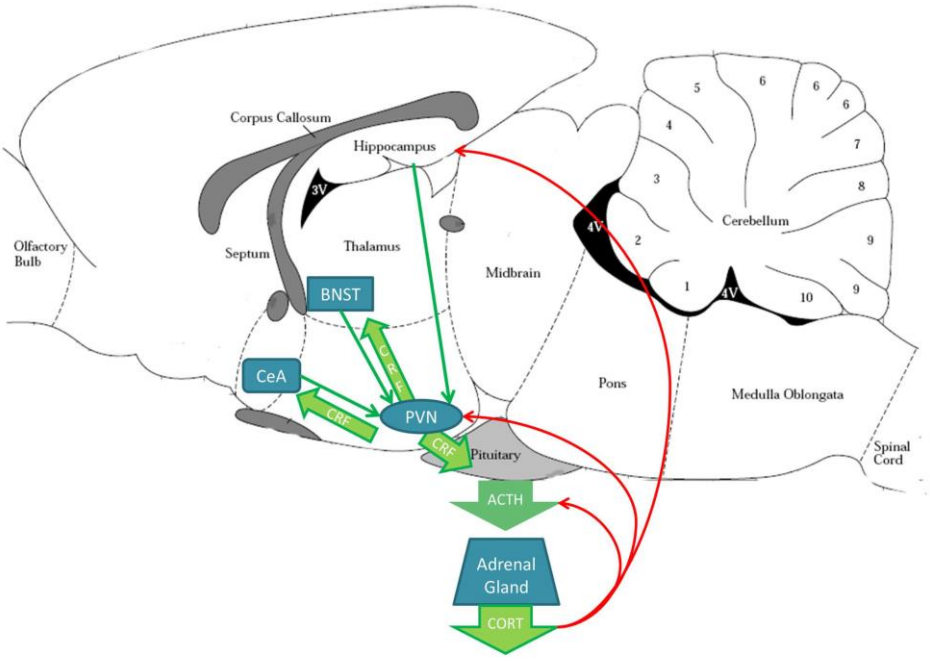


Figure 32. The possible brain regions and pathways involved in alcohol intoxication and withdrawal [112].

6. CONCLUSIONS

Alcohol addiction has three stages, including binge or intoxication, withdrawal or negative affect, and craving or preoccupation and anticipation.

Our study demonstrates for the first time that a single session of binge drinking produces anxiolytic and antidepressant effects immediately after binge drinking, rather than inducing anxiety-like and depression-like behavior, which resembles more closely how alcohol acts on humans. Furthermore, we were the first to investigate the role of CRF receptors in the affective component of binge drinking, and to demonstrate that the anxiolytic and antidepressant effects produced by binge drinking are mediated by CRF2, whereas the anxiety-like and depression-like signs observed the next day are promoted by CRF1, that might have clinical implications.

Also, our study demonstrates for the first time that the neuroendocrine changes induced by alcohol intoxication and withdrawal are mediated by CRF1, not CRF2, excepting the changes of hypothalamic AVP, which are not mediated by CRF receptors. Therefore, we provide a new evidence that 4-day ip administration of alcohol followed by 1-day abstinence in rats is a valid model for alcohol intoxication and withdrawal, characterized by specific changes of hypothalamic neurohormones and extrahypothalamic neurotransmitters, that could be used for therapeutical purposes.

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REFERENCES

1. Bale, T.L., K.F. Lee, and W.W. Vale, The role of corticotropin-releasing factor receptors in stress and anxiety. Integr Comp Biol, 2002. 42(3): p. 552-5.
2. Bale, T.L. and W.W. Vale, CRF and CRF receptors: role in stress responsivity and other behaviors. Annu Rev Pharmacol Toxicol, 2004. 44: p. 525-57.
3. Chang, C.P., et al., Identification of a seven transmembrane helix receptor for corticotropin-releasing factor and sauvagine in mammalian brain. Neuron, 1993. 11(6): p. 1187-95.
4. Lovenberg, T.W., et al., Cloning and characterization of a functionally distinct corticotropin-releasing factor receptor subtype from rat brain. Proc Natl Acad Sci U S A, 1995. 92(3): p. 836-40.
5. Eckart, K., et al., Pharmacology and biology of corticotropin-releasing factor (CRF) receptors. Receptors Channels, 2002. 8(3-4): p. 163-77.
6. Grammatopoulos, D.K., et al., Rat cerebral cortex corticotropin-releasing hormone receptors: evidence for receptor coupling to multiple G-proteins. J Neurochem, 2001. 76(2): p. 509-19.
7. Janssen, D. and T. Kozicz, Is it really a matter of simple dualism? Corticotropin-releasing factor receptors in body and mental health. Front Endocrinol (Lausanne), 2013. 4: p. 28.
8. Henckens, M.J., J.M. Deussing, and A. Chen, Region-specific roles of the corticotropin-releasing factor-urocortin system in stress. Nat Rev Neurosci, 2016. 17(10): p. 636-51.
9. Koob, G.F. and M. Le Moal, Drug addiction, dysregulation of reward, and allostasis. Neuropsychopharmacology, 2001. 24(2): p. 97-129.
10. de Kloet, E.R., M. Joels, and F. Holsboer, Stress and the brain: from adaptation to disease. Nat Rev Neurosci, 2005. 6(6): p. 463-75.
11. Dautzenberg, F.M. and R.L. Hauger, The CRF peptide family and their receptors: yet more partners discovered. Trends Pharmacol Sci, 2002. 23(2): p. 71-7.
12. Vaughan, J., et al., Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. Nature, 1995. 378(6554): p. 287-92.
13. Reul, J.M. and F. Holsboer, Corticotropin-releasing factor receptors 1 and 2 in anxiety and depression. Curr Opin Pharmacol, 2002. 2(1): p. 23-33.

14. Fekete, E.M. and E.P. Zorrilla, Physiology, pharmacology, and therapeutic relevance of urocortins in mammals: ancient CRF paralogs. Front Neuroendocrinol, 2007. 28(1): p. 1-27.
15. Suda, T., et al., Physiological roles of urocortins, human homologues of fish urotensin I, and their receptors. Peptides, 2004. 25(10): p. 1689-701.
16. Reyes, T.M., et al., Urocortin II: a member of the corticotropin-releasing factor (CRF) neuropeptide family that is selectively bound by type 2 CRF receptors. Proc Natl Acad Sci U S A, 2001. 98(5): p. 2843-8.
17. Lewis, K., et al., Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor. Proc Natl Acad Sci U S A, 2001. 98(13): p. 7570-5.
18. Hsu, S.Y. and A.J. Hsueh, Human stresscopin and stresscopin-related peptide are selective ligands for the type 2 corticotropin-releasing hormone receptor. Nat Med, 2001. 7(5): p. 605-11.
19. Hernandez, J.F., et al., Synthesis and relative potencies of new constrained CRF antagonists. J Med Chem, 1993. 36(20): p. 2860-7.
20. Morimoto, A., et al., The central role of corticotrophin-releasing factor (CRF-41) in psychological stress in rats. J Physiol, 1993. 460: p. 221-9.
21. Rivier, J., C. Rivier, and W. Vale, Synthetic competitive antagonists of corticotropin-releasing factor: effect on ACTH secretion in the rat. Science, 1984. 224(4651): p. 889-91.
22. Spina, M.G., et al., Behavioral effects of central administration of the novel CRF antagonist astressin in rats. Neuropsychopharmacology, 2000. 22(3): p. 230-9.
23. Deak, T., et al., The impact of the nonpeptide corticotropin-releasing hormone antagonist antalarmin on behavioral and endocrine responses to stress. Endocrinology, 1999. 140(1): p. 79-86.
24. Schulz, D.W., et al., CP-154,526: a potent and selective nonpeptide antagonist of corticotropin releasing factor receptors. Proc Natl Acad Sci U S A, 1996. 93(19): p. 10477-82.
25. Seymour, P.A., A.W. Schmidt, and D.W. Schulz, The pharmacology of CP-154,526, a non-peptide antagonist of the CRH1 receptor: a review. CNS Drug Rev, 2003. 9(1): p. 57-96.
26. Valdez, G.R., Development of CRF1 receptor antagonists as antidepressants and anxiolytics: progress to date. CNS Drugs, 2006. 20(11): p. 887-96.

27. Webster, E.L., et al., *In vivo* and *in vitro* characterization of antalarmin, a nonpeptide corticotropin-releasing hormone (CRH) receptor antagonist: suppression of pituitary ACTH release and peripheral inflammation. Endocrinology, 1996. 137(12): p. 5747-50.
28. Heinrichs, S.C. and Y. Tache, Therapeutic potential of CRF receptor antagonists: a gut-brain perspective. Expert Opin Investig Drugs, 2001. 10(4): p. 647-59.
29. Martinez, V., J. Rivier, and Y. Tache, Peripheral injection of a new corticotropin-releasing factor (CRF) antagonist, astressin, blocks peripheral CRF- and abdominal surgery-induced delayed gastric emptying in rats. J Pharmacol Exp Ther, 1999. 290(2): p. 629-34.
30. Martinez, V., et al., Central injection of a new corticotropin-releasing factor (CRF) antagonist, astressin, blocks CRF- and stress-related alterations of gastric and colonic motor function. J Pharmacol Exp Ther, 1997. 280(2): p. 754-60.
31. Rivier, J., et al., Potent and long-acting corticotropin releasing factor (CRF) receptor 2 selective peptide competitive antagonists. J Med Chem, 2002. 45(21): p. 4737-47.
32. Spierling, S.R. and E.P. Zorrilla, Don't stress about CRF: assessing the translational failures of CRF(1)antagonists. Psychopharmacology (Berl), 2017. 234(9-10): p. 1467-1481.
33. Pomrenze, M.B., et al., The Corticotropin Releasing Factor Receptor 1 in Alcohol Use Disorder: Still a Valid Drug Target? Alcohol Clin Exp Res, 2017. 41(12): p. 1986-1999.
34. Tache, Y. and B. Bonaz, Corticotropin-releasing factor receptors and stress-related alterations of gut motor function. J Clin Invest, 2007. 117(1): p. 33-40.
35. Kuperman, Y. and A. Chen, Urocortins: emerging metabolic and energy homeostasis perspectives. Trends Endocrinol Metab, 2008. 19(4): p. 122-9.
36. Bruijnzeel, A.W. and M.S. Gold, The role of corticotropin-releasing factor-like peptides in cannabis, nicotine, and alcohol dependence. Brain Res Brain Res Rev, 2005. 49(3): p. 505-28.
37. Sarnyai, Z., Y. Shaham, and S.C. Heinrichs, The role of corticotropin-releasing factor in drug addiction. Pharmacol Rev, 2001. 53(2): p. 209-43.
38. Volkow, N.D., G.F. Koob, and A.T. McLellan, Neurobiologic Advances from the Brain Disease Model of Addiction. N Engl J Med, 2016. 374(4): p. 363-71.
39. Chung, T., et al., Adolescent Binge Drinking. Alcohol Res, 2018. 39(1): p. 5-15.

40. van Schrojenstein Lantman, M., et al., Development of a Definition for the Alcohol Hangover: Consumer Descriptions and Expert Consensus. Curr Drug Abuse Rev, 2016. 9(2): p. 148-154.
41. Koob, G.F., Theoretical frameworks and mechanistic aspects of alcohol addiction: alcohol addiction as a reward deficit disorder. Curr Top Behav Neurosci, 2013. 13: p. 3-30.
42. Koob, G.F., Neurocircuitry of alcohol addiction: synthesis from animal models. Handb Clin Neurol, 2014. 125: p. 33-54.
43. Koob, G.F. and N.D. Volkow, Neurocircuitry of addiction. Neuropsychopharmacology, 2010. 35(1): p. 217-38.
44. Griebel, G. and F. Holsboer, Neuropeptide receptor ligands as drugs for psychiatric diseases: the end of the beginning? Nat Rev Drug Discov, 2012. 11(6): p. 462-78.
45. Carrasco, G.A. and L.D. Van de Kar, Neuroendocrine pharmacology of stress. Eur J Pharmacol, 2003. 463(1-3): p. 235-72.
46. Tsigos, C. and G.P. Chrousos, Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. J Psychosom Res, 2002. 53(4): p. 865-71.
47. Paxinos, G. and K.B.J. Franklin, The mouse brain in stereotaxic coordinates. Compact 2nd ed. 2004, Amsterdam ; Boston: Elsevier Academic Press.
48. Rhodes, J.S., et al., Evaluation of a simple model of ethanol drinking to intoxication in C57BL/6J mice. Physiol Behav, 2005. 84(1): p. 53-63.
49. Bagosi, Z., et al., The effects of CRF and urocortins on the preference for social novelty of mice. Behav Brain Res, 2017. 324: p. 146-154.
50. Bagosi, Z., et al., The effects of CRF and urocortins on the sociability of mice. Brain Res, 2017. 1663: p. 114-122.
51. Silvestre de Ferron, B., et al., Two Binges of Ethanol a Day Keep the Memory Away in Adolescent Rats: Key Role for GLUN2B Subunit. Int J Neuropsychopharmacol, 2015. 19(1).
52. Buzas, A., et al., Changes in striatal dopamine release and locomotor activity following acute withdrawal from chronic nicotine are mediated by CRF1, but not CRF2, receptors. Brain Res, 2019. 1706: p. 41-47.
53. Bagosi, Z., et al., Selective CRF2 receptor agonists ameliorate the anxiety- and depression-like state developed during chronic nicotine treatment and consequent acute withdrawal in mice. Brain Res, 2016. 1652: p. 21-29.

54. Lister, R.G., The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology (Berl), 1987. 92(2): p. 180-5.
55. Porsolt, R.D., A. Bertin, and M. Jalfre, Behavioral despair in mice: a primary screening test for antidepressants. Arch Int Pharmacodyn Ther, 1977. 229(2): p. 327-36.
56. Crawley, J.N., et al., Social approach behaviors in oxytocin knockout mice: comparison of two independent lines tested in different laboratory environments. Neuropeptides, 2007. 41(3): p. 145-63.
57. Kaidanovich-Beilin, O., et al., Assessment of social interaction behaviors. J Vis Exp, 2011(48).
58. Thiele, T.E. and M. Navarro, "Drinking in the dark" (DID) procedures: a model of binge-like ethanol drinking in non-dependent mice. Alcohol, 2014. 48(3): p. 235-41.
59. Thiele, T.E., J.C. Crabbe, and S.L. Boehm, 2nd, "Drinking in the Dark" (DID): a simple mouse model of binge-like alcohol intake. Curr Protoc Neurosci, 2014. 68: p. 9 49 1-12.
60. Paxinos, G. and C. Watson, The rat brain in stereotaxic coordinates. 6th ed. 2007, Amsterdam ; Boston ;: Academic Press/Elsevier.
61. Suda, T., et al., *In vitro* study of immunoreactive corticotropin-releasing factor release from the rat hypothalamus. Life Sci, 1985. 37(16): p. 1499-505.
62. Joanny, P., et al., Corticotropin-releasing factor release from *in vitro* superfused and incubated rat hypothalamus. Effect of potassium, norepinephrine, and dopamine. Peptides, 1989. 10(5): p. 903-11.
63. Purves, H.D. and N.E. Sirett, Assay of corticotrophin in dexamethasone-treated rats. Endocrinology, 1965. 77(2): p. 366-74.
64. Zenker, N. and D.E. Bernstein, The estimation of small amounts of corticosterone in rat plasma. J Biol Chem, 1958. 231(2): p. 695-701.
65. Gaddum, J.H., The technique of superfusion. Br J Pharmacol Chemother, 1953. 8(3): p. 321-6.
66. Harsing, L.G., Jr. and E.S. Vizi, Release of endogenous dopamine from rat isolated striatum: effect of clorgyline and (-)-deprenyl. Br J Pharmacol, 1984. 83(3): p. 741-9.
67. Olney, J.J., S.A. Marshall, and T.E. Thiele, Assessment of depression-like behavior and anhedonia after repeated cycles of binge-like ethanol drinking in male C57BL/6J mice. Pharmacol Biochem Behav, 2018. 168: p. 1-7.

68. Evans, O., et al., Alcohol Binge Drinking and Anxiety-Like Behavior in Socialized *Versus* Isolated C57BL/6J Mice. Alcohol Clin Exp Res, 2020. 44(1): p. 244-254.
69. Van Hees, L., et al., Voluntary alcohol binge-drinking in adolescent C57Bl6 mice induces delayed appearance of behavioural defects in both males and females. Addict Biol, 2022. 27(1): p. e13102.
70. Lopez-Cruz, L., et al., Ethanol and Caffeine Effects on Social Interaction and Recognition in Mice: Involvement of Adenosine A(2A) and A(1) Receptors. Front Behav Neurosci, 2016. 10: p. 206.
71. Lopez-Cruz, L., J.D. Salamone, and M. Correa, The Impact of Caffeine on the Behavioral Effects of Ethanol Related to Abuse and Addiction: A Review of Animal Studies. J Caffeine Res, 2013. 3(1): p. 9-21.
72. Marsland, P., et al., Male, but not female, Sprague Dawley rats display enhanced fear learning following acute ethanol withdrawal (hangover). Pharmacol Biochem Behav, 2021. 208: p. 173229.
73. Palmer, E., et al., Alcohol Hangover: Underlying Biochemical, Inflammatory and Neurochemical Mechanisms. Alcohol Alcohol, 2019. 54(3): p. 196-203.
74. Karadayian, A.G., et al., Alterations in affective behavior during the time course of alcohol hangover. Behav Brain Res, 2013. 253: p. 128-38.
75. Karadayian, A.G. and R.A. Cutrera, Alcohol hangover: type and time-extension of motor function impairments. Behav Brain Res, 2013. 247: p. 165-73.
76. Lee, K.M., et al., Binge alcohol drinking elicits persistent negative affect in mice. Behav Brain Res, 2015. 291: p. 385-398.
77. Lee, K.M., et al., Negative Affect and Excessive Alcohol Intake Incubate during Protracted Withdrawal from Binge-Drinking in Adolescent, But Not Adult, Mice. Front Psychol, 2017. 8: p. 1128.
78. Jimenez Chavez, C.L., et al., Incubation of Negative Affect during Protracted Alcohol Withdrawal Is Age-, but Not Sex-Selective. Brain Sci, 2020. 10(6).
79. Jimenez Chavez, C.L., et al., Alcohol-Drinking Under Limited-Access Procedures During Mature Adulthood Accelerates the Onset of Cognitive Impairment in Mice. Front Behav Neurosci, 2022. 16: p. 732375.
80. Kaczmarek, L., Bed Nucleus of the Stria Terminalis-Derived Corticotropin-Releasing Factor Controls Binge Alcohol Drinking Via Interacting With Corticotropin-Releasing Factor Receptors 1 and 2 in the Ventral Tegmental Area. Biol Psychiatry, 2017. 81(11): p. 905-906.

81. Albrechet-Souza, L., et al., Corticotropin Releasing Factor Binding Protein and CRF2 Receptors in the Ventral Tegmental Area: Modulation of Ethanol Binge Drinking in C57BL/6J Mice. Alcohol Clin Exp Res, 2015. 39(9): p. 1609-18.
82. Sparta, D.R., et al., Binge ethanol-drinking potentiates corticotropin releasing factor R1 receptor activity in the ventral tegmental area. Alcohol Clin Exp Res, 2013. 37(10): p. 1680-7.
83. Kaur, S., et al., Corticotropin-releasing factor acting on corticotropin-releasing factor receptor type 1 is critical for binge alcohol drinking in mice. Alcohol Clin Exp Res, 2012. 36(2): p. 369-76.
84. Budygin, E.A., et al., Chronic ethanol exposure alters presynaptic dopamine function in the striatum of monkeys: a preliminary study. Synapse, 2003. 50(3): p. 266-8.
85. Budygin, E.A., et al., Effect of acute ethanol on striatal dopamine neurotransmission in ambulatory rats. J Pharmacol Exp Ther, 2001. 297(1): p. 27-34.
86. Vengeliene, V., et al., Neuropharmacology of alcohol addiction. Br J Pharmacol, 2008. 154(2): p. 299-315.
87. Herman, M.A., et al., Enhanced GABAergic transmission in the central nucleus of the amygdala of genetically selected Marchigian Sardinian rats: alcohol and CRF effects. Neuropharmacology, 2013. 67: p. 337-48.
88. McBride, W.J., Central nucleus of the amygdala and the effects of alcohol and alcohol-drinking behavior in rodents. Pharmacol Biochem Behav, 2002. 71(3): p. 509-15.
89. Diaz, M.R. and R.A. Morton, Ethanol untangles the amygdala-anxiety circuit through tonic GABA inhibition. Alcohol Clin Exp Res, 2014. 38(3): p. 619-23.
90. Roberto, M., et al., Increased GABA release in the central amygdala of ethanol-dependent rats. J Neurosci, 2004. 24(45): p. 10159-66.
91. Roberto, M., N.W. Gilpin, and G.R. Siggins, The central amygdala and alcohol: role of gamma-aminobutyric acid, glutamate, and neuropeptides. Cold Spring Harb Perspect Med, 2012. 2(12): p. a012195.
92. Chefer, V., et al., Repeated exposure to moderate doses of ethanol augments hippocampal glutamate neurotransmission by increasing release. Addict Biol, 2011. 16(2): p. 229-37.
93. Martin, D. and H.S. Swartzwelder, Ethanol inhibits release of excitatory amino acids from slices of hippocampal area CA1. Eur J Pharmacol, 1992. 219(3): p. 469-72.

94. Sepulveda, C., et al., Effects of *in vitro* ethanol and chronic ethanol consumption on the release of excitatory amino acids in the rat hippocampus. Brain Res, 1995. 674(1): p. 104-6.
95. Bale, T.L., CRF as the key component of stress response systems. Front Neuroendocrinol, 2014. 35(2): p. 159-60.
96. Lowery, E.G., et al., CRF-1 antagonist and CRF-2 agonist decrease binge-like ethanol drinking in C57BL/6J mice independent of the HPA axis. Neuropsychopharmacology, 2010. 35(6): p. 1241-52.
97. Rivier, C., Role of hypothalamic corticotropin-releasing factor in mediating alcohol-induced activation of the rat hypothalamic-pituitary-adrenal axis. Front Neuroendocrinol, 2014. 35(2): p. 221-33.
98. Rivier, C., T. Bruhn, and W. Vale, Effect of ethanol on the hypothalamic-pituitary-adrenal axis in the rat: role of corticotropin-releasing factor (CRF). J Pharmacol Exp Ther, 1984. 229(1): p. 127-31.
99. Rivier, C., J. Rivier, and W. Vale, Inhibition of adrenocorticotropic hormone secretion in the rat by immunoneutralization of corticotropin-releasing factor. Science, 1982. 218(4570): p. 377-9.
100. Rivier, C. and W. Vale, Modulation of stress-induced ACTH release by corticotropin-releasing factor, catecholamines and vasopressin. Nature, 1983. 305(5932): p. 325-7.
101. Ogilvie, K.M., S. Lee, and C. Rivier, Role of arginine vasopressin and corticotropin-releasing factor in mediating alcohol-induced adrenocorticotropin and vasopressin secretion in male rats bearing lesions of the paraventricular nuclei. Brain Res, 1997. 744(1): p. 83-95.
102. Rivest, S. and C. Rivier, Lesions of hypothalamic PVN partially attenuate stimulatory action of alcohol on ACTH secretion in rats. Am J Physiol, 1994. 266(2 Pt 2): p. R553-8.
103. Rivier, C. and S. Lee, Acute alcohol administration stimulates the activity of hypothalamic neurons that express corticotropin-releasing factor and vasopressin. Brain Res, 1996. 726(1-2): p. 1-10.
104. Ogilvie, K., S. Lee, and C. Rivier, Effect of three different modes of alcohol administration on the activity of the rat hypothalamic-pituitary-adrenal axis. Alcohol Clin Exp Res, 1997. 21(3): p. 467-76.
105. Rivier, C., Gender, sex steroids, corticotropin-releasing factor, nitric oxide, and the HPA response to stress. Pharmacol Biochem Behav, 1999. 64(4): p. 739-51.

106. Lee, S. and C. Rivier, Alcohol increases the expression of type 1, but not type 2 alpha corticotropin-releasing factor (CRF) receptor messenger ribonucleic acid in the rat hypothalamus. Brain Res Mol Brain Res, 1997. 52(1): p. 78-89.
107. Rivier, C.L., D.E. Grigoriadis, and J.E. Rivier, Role of corticotropin-releasing factor receptors type 1 and 2 in modulating the rat adrenocorticotropin response to stressors. Endocrinology, 2003. 144(6): p. 2396-403.
108. Bagosi, Z., et al., The effects of corticoptropin-releasing factor and the urocortins on striatal dopamine release induced by electrical stimulation-an *in vitro* superfusion study. Neurochem Res, 2006. 31(2): p. 209-13.
109. Bagosi, Z., et al., The effects of CRF and the urocortins on [3H]GABA release from the rat amygdala--an *in vitro* superfusion study. Brain Res Bull, 2008. 75(1): p. 15-7.
110. Bagosi, Z., et al., The effects of CRF and urocortins on the hippocampal glutamate release. Neurochem Int, 2015. 90: p. 67-71.
111. Pinter, D., et al., The effects of CRF and the urocortins on the hippocampal acetylcholine release in rats. Neuropeptides, 2021. 88: p. 102147.
112. Rutherford, H.J., et al., Disruption of maternal parenting circuitry by addictive process: rewiring of reward and stress systems. Front Psychiatry, 2011. 2: p. 37.

APPENDIX

biomedicines

MDPI

Article

The Effects of Alcohol Intoxication and Withdrawal on Hypothalamic Neurohormones and Extrahypothalamic Neurotransmitters

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Abstract: The aim of the present study was to determine the effects of alcohol intoxication and withdrawal on hypothalamic neurohormones such as corticotropin-releasing factor (CRF) and arginine vasopressin (AVP), and extrahypothalamic neurotransmitters such as striatal dopamine (DA), amygdalar gamma aminobutyric acid (GABA), and hippocampal glutamate (GLU). In addition, the participation of the two CRF receptors, CRF1 and CRF2, was investigated. For this purpose, male Wistar rats were exposed to repeated intraperitoneal (ip) administration of alcohol every 12 h, for 4 days and then for 1 day of alcohol abstinence. On the fifth or sixth day, intracerebroventricular (icv) administration of selective CRF1 antagonist antalarmin or selective CRF2 antagonist astressin$_2$B was performed. After 30 min, the expression and concentration of hypothalamic CRF and AVP, the concentration of plasma adrenocorticotropic hormone (ACTH) and corticosterone (CORT), and the release of striatal DA, amygdalar GABA, and hippocampal GLU were measured. Our results indicate that the neuroendocrine changes induced by alcohol intoxication and withdrawal are mediated by CRF1, not CRF2, except for the changes in hypothalamic AVP, which are not mediated by CRF receptors.

Keywords: alcohol intoxication; alcohol withdrawal; CRF; AVP; dopamine; GABA; glutamate

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1. Introduction

Alcohol addiction has three stages including binge/intoxication, withdrawal/negative affect, and preoccupation/anticipation (craving). Each stage is characterized by specific changes in the hypothalamic neurohormones such as corticotropin-releasing factor (CRF) and arginine vasopressin (AVP), as well as extrahypothalamic neurotransmitters such as striatal dopamine (DA), amygdalar gamma aminobutyric acid (GABA), and hippocampal glutamate (GLU) [1,2].

The stage of binge/intoxication is associated with the activation of the hypothalamic–pituitary–adrenal (HPA) axis that is initiated by hypothalamic CRF and/or AVP [3]. The HPA axis consists of the paraventricular nucleus of the hypothalamus (PVN), the anterior pituitary, and the adrenal cortex, and can be stimulated by various stressors [4,5]. Alcohol can also stimulate the release of CRF and/or AVP from the PVN, which, in turn, evokes the release of adrenocorticotrop hormone (ACTH) from the anterior pituitary. Subsequently, pituitary ACTH stimulates the release of glucocorticoids in the adrenal cortex, which are represented mainly by cortisol in humans and corticosterone (CORT) in rodents. The elevation of the plasma ACTH and glucocorticoid levels not only reflects the activation of

the HPA axis, but also exerts a negative feedback effect on the release of hypothalamic CRF and/or AVP, thereby inhibiting the HPA axis [4,5].

The stage of withdrawal/negative affect is associated with the activation of the extended amygdala circuit, which is mediated by extrahypothalamic CRF and norepinephrine [3]. The extended amygdala circuit consists of the central nucleus of the amygdala (CeA), the bed nucleus of stria terminalis (BNST), and the shell of the nucleus accumbens (shNAcc), and represents an interface between the reward and stress systems [4,5]. During alcohol intoxication, the reward system is activated [3]. Alcohol stimulates the DA release in the striatum, and the GABA release in the amygdala, inducing rewarding, anxiolytic, and antidepressant effects [3]. Alcohol intoxication may also result in amnesia, which can be related to the reduction in the hippocampal GLU release [3]. During alcohol withdrawal, as an anti-reward mechanism, the stress system is activated, resulting in alcohol withdrawal syndrome, which includes symptoms such as anhedonia, anxiety, and aggression [3]. These symptoms can be explained by the reduction in the striatal DA and amygdalar GABA release, and the stimulation of GLU release in the hippocampus [3].

Alcohol withdrawal syndrome consists of somatic (physical) signs and affective (emotional) symptoms that emerge immediately after alcohol cessation [1,2]. The physical signs usually cease within 24 h following alcohol intoxication (acute alcohol withdrawal), whereas the emotional symptoms may persist for days to years (protracted alcohol withdrawal), producing craving that makes one vulnerable to relapse, especially in periods of stress [1,2]. This last stage of preoccupation/anticipation (craving) is associated with the activation of the hippocampus, orbitofrontal cortex, prefrontal cortex, insula and basolateral amygdala (BLA), and is believed to be mediated by both hypothalamic and extrahypothalamic CRF [3].

CRF acts via two distinct CRF receptors, CRF1 and CRF2, with putatively antagonistic actions in the central nervous system (CNS) [6]. CRF1 is distributed predominantly in the cerebral cortex, anterior pituitary, and cerebellum, but it is also expressed in the striatum, amygdala, and hippocampus [7], and seems to promote activation of the HPA axis, anxiety, and depression [8–10]. In contrast, CRF2 is limited centrally to the subcortical regions including the striatum, amygdala, and hippocampus [7] and appears to mediate anxiolytic and antidepressant effects [8–10].

The aim of our study was to determine the effects of alcohol intoxication and withdrawal on hypothalamic neurohormones and extrahypothalamic neurotransmitters in rats. Since CRF was involved in all three stages of alcohol addiction, the participation of the two CRF receptors was investigated. For this purpose, male Wistar rats were exposed to repeated intraperitoneal (ip) administration of alcohol every 12 h, for 4 days and then for 1 day of alcohol withdrawal. On the fifth day (immediately after the last ip administration of alcohol) or the sixth day (24 h after the last ip administration of alcohol), intracerebroventricular (icv) administration of the selective CRF1 antagonist antalarmin or selective CRF2 antagonist astressin$_2$B was performed. After 30 min, the mice were decapitated without anesthesia, trunk blood was collected, and the brains were removed. From the brain, the expression and concentration of hypothalamic CRF and AVP as well as the release of striatal DA, amygdalar GABA, and hippocampal GLU were determined. From the trunk blood, the concentration of plasma ACTH and CORT were measured.

2. Materials and Methods

2.1. Animals

Male Wistar rats weighing 150–250 g were used (N = 72). The rats were treated in accordance with the ARRIVE guidelines and the experiments were carried out in concordance with the EU Directive 2010/63/EU for animal experiments. They were housed together and kept in their home cages at a constant temperature on a standard illumination schedule with 12-h light and 12-h dark periods (lights on from 6:00). Commercial food and tap water were available ad libitum. They were also handled daily in order to minimize the effects of non-specific stress.

2.2. Substances

The saline and alcohol solutions administered ip were provided by B. Braun Inc., Melsungen, Germany and Reanal Ltd., Budapest, Hungary, respectively. The selective CRF1 antagonist antalarmin and the selective CRF2 antagonist astressin$_2$B administered via icv administration were ordered from Sigma-Aldrich Inc., St. Louis, MO, USA. The acetic acid used for the in vitro homogenization was provided by Reanal Ltd., Budapest, Hungary. Ethanol was determined from the plasma by commercially available enzymatic kit (Roche Diagnostics, Mannheim, Germany) on cobas c502 analyzer (Roche Diagnostics, Mannheim, Germany). The sensitivity of the assay was 10.1 mg/dL (0.01 g/dL). The GeneJET RNA Purification Kit, the Maxima First Strand cDNA Synthesis Kit, and the NanoDrop One device required for the determination of hypothalamic CRF and AVP expression was provided by Thermo Scientific Inc., Waltham, MA, USA. The sandwich Enzyme-linked Immunosorbent Assay (ELISA) Kits required for the determination of the hypothalamic CRF and AVP, and plasma ACTH concentrations were purchased from Phoenix Pharmaceuticals Ltd., Mannheim, Germany. The methylene chloride, sulfuric acid, and ethyl-alcohol solutions used for the determination of the plasma CORT concentration were provided by Reanal Ltd., Budapest, Hungary. The Krebs solution was provided by Reanal Ltd., Hungary. The tritium-labelled neurotransmitters including the [^{3}H]DA, [^{3}H]GABA, and [^{3}H]GLU, and the Ultima Gold scintillation fluid used for the in vitro superfusion studies and liquid scintillation were ordered from Perkin-Elmer Inc., Waltham, MA, USA.

2.3. Surgery

The rats were implanted with a stainless steel Luer cannula (10 mm long) that was aimed at the right lateral cerebral ventricle. The surgical intervention was performed under anesthesia with 35 mg/kg pentobarbital sodium (Euthanasol, CEVA-Phylaxia, Budapest, Hungary). The stereotaxic coordinates for the right lateral cerebral ventricle were 0.2 mm posterior and 1.7 mm lateral to the bregma, and 3.7 mm deep from the dural surface, according to a stereotaxic atlas of the rat brain [11]. Cannulas were secured to the skull with dental cement and acrylate. The rats were allowed to recover for 7 days after surgery.

2.4. Treatment

Male Wistar rats were exposed to repeated ip administration of alcohol every 12 h, for 4 days and then for 1 day of alcohol withdrawal. The protocol of alcohol administration was based on a previous study in which 20% alcohol was administered at dose of 3 g/kg [12] (Table 1).

Table 1. The treatment protocol.

Group	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Group #1 (N = 6)	Saline ip at 8:00 and 20:00	Saline ip at 8:00 and 20:00	Saline ip at 8:00 and 20:00	Saline ip at 8:00 and 20:00	Saline ip + saline icv at 8:00 + assays at 8:30	
Group #2 (N = 6)	Saline ip at 8:00 and 20:00	Saline ip at 8:00 and 20:00	Saline ip at 8:00 and 20:00	Saline ip at 8:00 and 20:00	Saline ip + antalarmin icv at 8:00 + assays at 8:30	
Group #3 (N = 6)	Saline ip at 8:00 and 20:00	Saline ip at 8:00 and 20:00	Saline ip at 8:00 and 20:00	Saline ip at 8:00 and 20:00	Saline ip + astressin$_2$B icv at 8:00 + assays at 8:30	

Table 1. *Cont.*

Group	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Group #4 (*N* = 6)	Alcohol ip at 8:00 and 20:00	Alcohol ip at 8:00 and 20:00	Alcohol ip at 8:00 and 20:00	Alcohol ip at 8:00 and 20:00	Alcohol ip + saline icv at 8:00 + assays at 8:30	
Group #4 (*N* = 6)	Alcohol ip at 8:00 and 20:00	Alcohol ip at 8:00 and 20:00	Alcohol ip at 8:00 and 20:00	Alcohol ip at 8:00 and 20:00	Alcohol ip + saline icv at 8:00 + assays at 8:30	
Group #5 (*N* = 6)	Alcohol ip at 8:00 and 20:00	Alcohol ip at 8:00 and 20:00	Alcohol ip at 8:00 and 20:00	Alcohol ip at 8:00 and 20:00	Alcohol ip + antalarmin icv at 8:00 + assays at 8:30	
Group #6 (*N* = 6)	Alcohol ip at 8:00 and 20:00	Alcohol ip at 8:00 and 20:00	Alcohol ip at 8:00 and 20:00	Alcohol ip at 8:00 and 20:00	Alcohol ip + astressin$_2$B icv at 8:00 + assays at 8:30	
Group #7 (*N* = 6)	Saline ip at 8:00 and 20:00	Saline ip at 8:00 and 20:00	Saline ip at 8:00 and 20:00	Saline ip at 8:00 and 20:00	Saline ip at 8:00	Saline icv at 8:00 + assays at 8:30
Group #8 (*N* = 6)	Saline ip at 8:00 and 20:00	Saline ip at 8:00 and 20:00	Saline ip at 8:00 and 20:00	Saline ip at 8:00 and 20:00	Saline ip at 8:00	Antalarmin icv at 8:00 + assays at 8:30
Group #9 (*N* = 6)	Saline ip at 8:00 and 20:00	Saline ip at 8:00 and 20:00	Saline ip at 8:00 and 20:00	Saline ip at 8:00 and 20:00	Saline ip at 8:00	Astressin$_2$B icv at 8:00 + assays at 8:30
Group #10 (*N* = 6)	Alcohol ip at 8:00 and 20:00	Alcohol ip at 8:00 and 20:00	Alcohol ip at 8:00 and 20:00	Alcohol ip at 8:00 and 20:00	Alcohol ip at 8:00	Saline icv at 8:00 + assays at 8:30
Group #11 (*N* = 6)	Alcohol ip at 8:00 and 20:00	Alcohol ip at 8:00 and 20:00	Alcohol ip at 8:00 and 20:00	Alcohol ip at 8:00 and 20:00	Alcohol ip at 8:00	Antalarmin icv at 8:00 + assays at 8:30
Group #12 (*N* = 6)	Alcohol ip at 8:00 and 20:00	Alcohol ip at 8:00 and 20:00	Alcohol ip at 8:00 and 20:00	Alcohol ip at 8:00 and 20:00	Alcohol ip at 8:00	Astressin$_2$B icv at 8:00 + assays at 8:30

This amount of alcohol produced a blood alcohol concentration (BAC) of 197.5 ± 19 mg/dL, measured at 30 min after the ip administration [12]. On the fifth (immediately after the last ip administration of alcohol) or sixth day (24 h after the last ip administration of alcohol), the rats were administered icv with 0.1 μg/2 μL of antalarmin or 1 μg/2 μL of astressin$_2$B. The doses of CRF1 and CRF2 antagonists were based on our previous studies, which indicated that these doses efficiently block the activation of the HPA axis and the striatal DA release observed during nicotine withdrawal [13,14]. After 30 min, the mice were decapitated without anesthesia, the trunk blood was collected, and the brains were removed. From the brain, the expression and concentration of hypothalamic CRF and AVP as well as the release of striatal DA, amygdalar GABA, and hippocampal GLU were determined. From the trunk blood, the concentration of plasma ACTH and CORT were measured.

2.5. Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR)

For the determination of the hypothalamic CRF and AVP expression, quantitative reverse transcription polymerase chain reaction (RT-qPCR) was performed. First, the rats were decapitated, their brains removed, and then dissected in a Petri dish filled with ice-cold Krebs solution. The hypothalamus was isolated from each rat according to a

stereotaxic atlas of the rat brain [11] after the following coordinates: rostro-caudal (RC) +2.6 to −2.6 mm, medio-lateral (ML) +1.5 to −1.5 mm, dorso-ventral (DV) +7 to +10 mm (Figure 1).

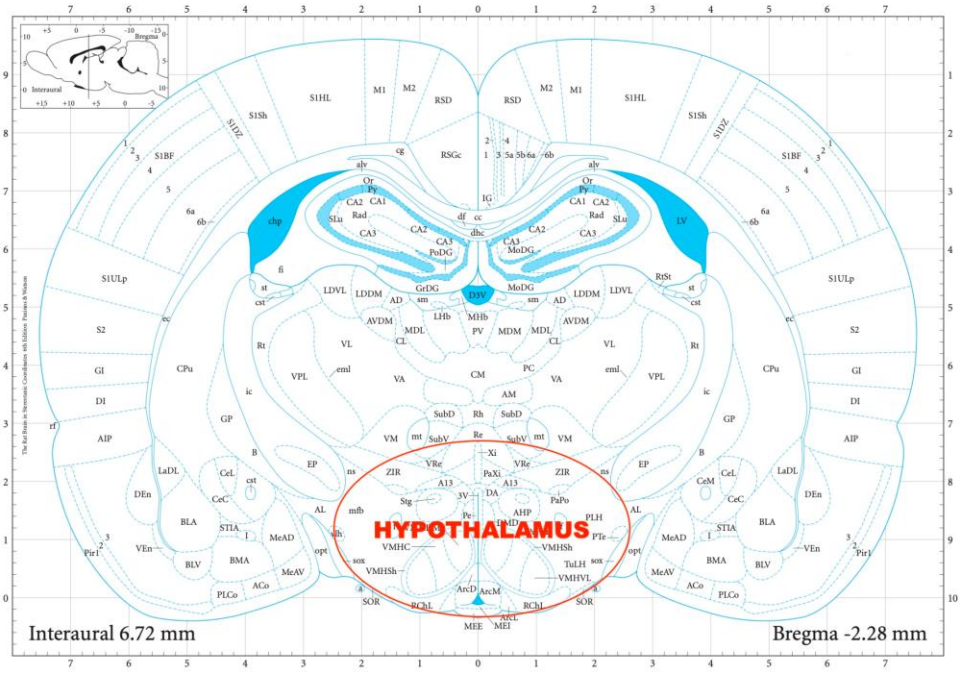

Figure 1. The dissection of the hypothalamus according to a stereotaxic atlas of the rat brain. Reprinted/adapted with permission from Ref. [11]. The coordinates were: rostro-caudal (RC) +2.6–−2.6 mm, medio-lateral (ML) +1.5–−1.5 mm, dorso-ventral (DV) +7–+10 mm for the hypothalamus.

The tissue samples were stored in 1 mL of TRIzol (Thermo Fisher Scientific, Waltham, MA, USA) in Eppendorf tubes and kept in a freezer at −80 °C and then underwent ultrasonic homogenization (Branson Sonifier 250, Emerson, St. Louis, MO, USA), then 200 µL of chloroform was added to each sample. Following 10 min of incubation at room temperature, the samples were centrifuged for 15 min at 13,000× *g* (Heraeus Fresco 17, Thermo Fisher Scientific, Waltham, MA, USA). Approximately 500 µL of the supernatant was collected from each tube and transferred to new Eppendorf tubes containing 600 µL of 96% alcohol that were stored overnight at −20 °C. On the following day, the GeneJET RNA Purification Kit (Thermo Fisher Scientific, USA) was used according to the manufacturer's instructions. The concentration of the purified samples was calculated based on the average of three measurements with a spectrophotometer (NanoDrop One, Thermo Scientific Inc., Waltham, MA, USA). The RNA samples were deemed uncontaminated if the 260/280 nm ratio was between 1.8 and 2.2. A volume containing 300 ng of RNA was obtained from each sample for cDNA synthesis. The first strand cDNA was synthesized using the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions.

The qPCR reaction mix was prepared using the Luminaris Color HiGreen Low ROX qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. A total volume of 10 µL of reaction mix was prepared containing 5 µL of Master Mix, 0.3 µL of forward primer, 0.3 µL of reverse primer, 1.67 µL of cDNA,

and 2.73 µL of nuclease-free water. The custom primers corresponding to the CRF, AVP, and GPADH genes are shown in Table 2.

Table 2. The custom primers.

Gene	Forward	Reverse
CRF	5′-TGG TGT GGA GAA ACT CAG AGC-3′	5′-CAT GTT AGG GGC GCT CTC TTC-3′
AVP	5′-CTG ACA TGG AGC TGA GAC AGT-3′	5′-CGC AGC TCT CGT CGC T-3′
Gapdh	5′-CGG CCA AAT CTG AGG CAA GA-3′	5′-TTT TGT GAT GCG TGT GTA GCG-3′

The mix was placed in a thermal cycler (C1000 Touch Thermal Cycler, BioRad, Budapest, Hungary) that was programmed according to the cycling protocol in Table 3. The expression of each gene relative to *Gapdh* was determined using the ΔΔCT method.

Table 3. The cycling protocol.

Phase	Temperature °C	Time	Number of Cycles
UDG pre-treatment	50	2 min	1
Initial denaturation	95	10 min	1
Denaturation	95	15 s	40
Annealing	60	30 s	40
Extension	72	30 s	40

2.6. Sandwich Enzyme-Linked Immunosorbent Assay (ELISA)

For the determination of the hypothalamic CRF and AVP and plasma ACTH concentrations an in vitro homogenization was performed [15,16], which was followed by the sandwich ELISA. First, the rats were decapitated, their brains removed, and then dissected in a Petri dish filled with ice-cold Krebs solution and their trunk blood collected. The hypothalamus was isolated from each rat according to the stereotaxic atlas of the rat brain, as previously described [11]. The samples were dissolved in 500 µL acetic acid at a 2 M concentration in Eppendorf tubes and immersed in boiling water for 5 min. Next, the samples were homogenized with an ultrasonic homogenizer (Branson Sonifier 250, Emerson, St. Louis, MO, USA) on ice for 30 s. The homogenates were centrifuged twice at 10,000 rpm at 4 °C for 20 min, after which the supernatants were separated and underwent lyophilization for further determinations. The hypothalamic CRF and AVP and plasma ACTH concentrations were determined according to the manufacturer's instructions (Phoenix Pharmaceuticals Ltd., Mannheim, Germany) and expressed as ng/mL.

2.7. Chemofluorescent Assay

For the determination of the plasma CORT concentration, a chemofluorescent assay was performed as described originally by Purves and Sirett, and modified later by Zenker and Bernstein [17,18]. All substances used, including methylene chloride, sulfuric acid, and ethyl-alcohol solutions, used for the determination of the plasma CORT concentration were provided by Reanal Ltd., Budapest, Hungary and the plasma CORT concentration was expressed as µg/100 mL.

2.8. In Vitro Superfusion Assay

The striatal DA, amygdalar GABA, and hippocampal GLU release were measured by means of an in vitro superfusion system, which was described originally by Gaddum, and later improved by Harsing and Vizi [19,20]. The rats were decapitated, their brains removed, and then dissected in a Petri dish filled with ice-cold Krebs solution. The striatum, amygdala, and hippocampus were isolated from each rat according to a stereotaxic atlas

of the rat brain [11] after the following coordinates: RC +4.0 to −1.0 mm, ML +1.0 to +5.0 mm, DV +3.0 to +8.0 mm for the striatum (Figure 2); RC 0.0 to −2.0 mm, ML +3.0 to +6.0 mm, DV +7.0 to +10.0 mm for the amygdala (Figure 3); and RC −4.0 to −6.0 mm, ML +2.0 to +5.0 mm, DV +3.0 to +8.0 mm for the hippocampus (Figure 4). The brain tissue was dissected, incubated for 30 min in 8 mL of Krebs solution with 15 mM of [^{3}H]DA, [^{3}H]GABA, or [^{3}H]GLU incubated, then superfused for 30 min and electrically stimulated for 2 min with the means of a superfusion system provided by Experimetria Ltd., Budapest, Hungary. The superfusates were collected in Eppendorf tubes by a multichannel fraction collector (Gilson FC 203B). The total collecting time was 32 min (4 × 16 samples, 2 min each) and the peak of the fractional release was observed at 14 min. In the corresponding figures, only these peaks were represented. Finally, the brain tissue was removed from the superfusion system and solubilized in Krebs solution using an ultrasonic homogenizer called a Branson Sonifier 250. After the addition of scintillation fluid to the samples and the remaining brain tissue, the radioactivity was measured with a liquid scintillation spectrometer (Tri-carb 2100TR, Packard Inc., Ramsey, MN, USA) and expressed in count per minute (CPM). The fractional release was calculated as the ratio between the radioactivity of the samples and that of the remaining brain tissue.

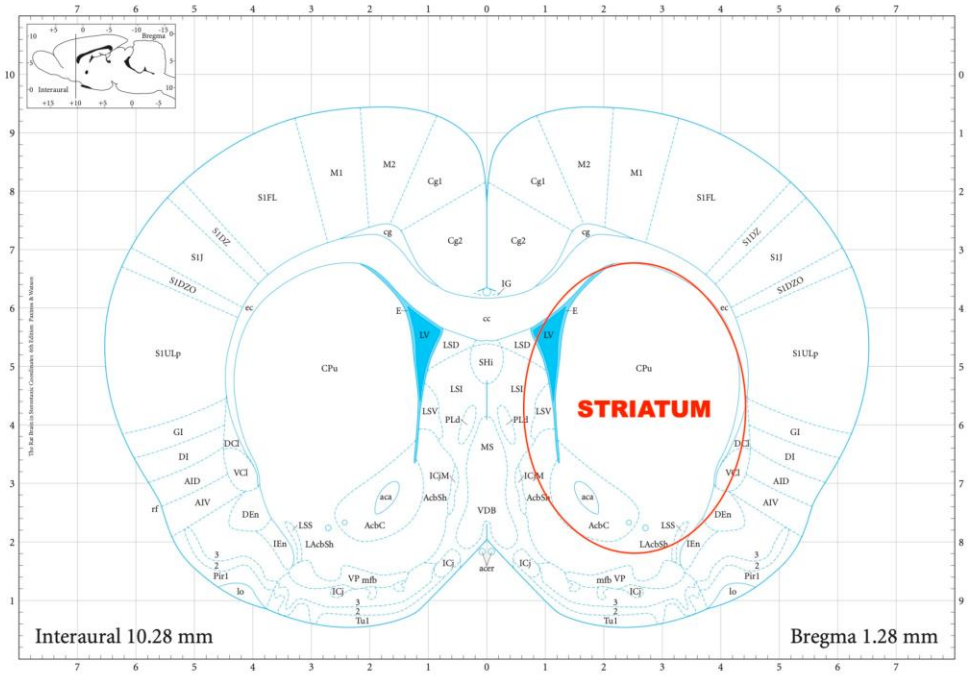

Figure 2. The dissection of the striatum according to a stereotaxic atlas of the rat brain. Reprinted/adapted with permission from Ref. [11]. The coordinates were: rostro-caudal (RC) +4.0–−1.0 mm, medio-lateral (ML) +1.0–+5.0 mm, dorso-ventral (DV) +3.0–+8.0 mm for the striatum.

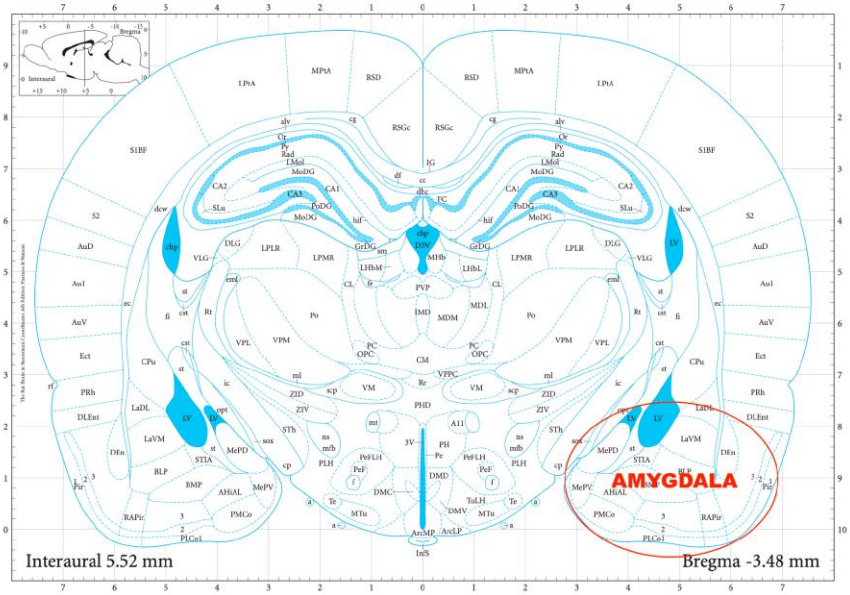

Figure 3. The dissection of the amygdala according to a stereotaxic atlas of the rat brain. Reprinted/adapted with permission from Ref. [11]. The coordinates were: rostro-caudal (RC) 0.0–−2.0 mm, medio-lateral (ML) +3.0–+6.0 mm, dorso-ventral (DV) +7.0–+10.0 mm for the amygdala.

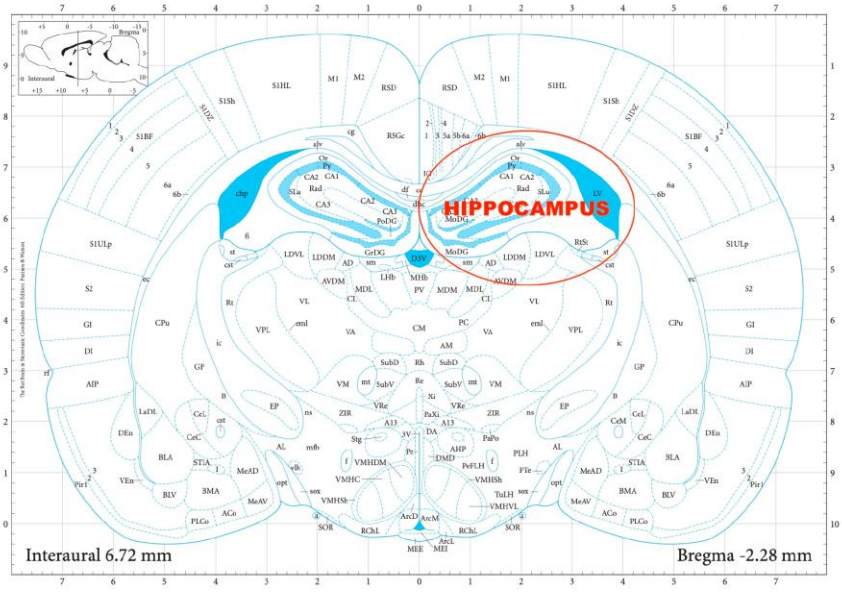

Figure 4. The dissection of the hippocampus according to a stereotaxic atlas of the rat brain. Reprinted/adapted with permission from Ref. [11]. The coordinates were: rostro-caudal (RC) −4.0–−6.0 mm, medio-lateral (ML) +2.0–+5.0 mm, dorso-ventral (DV) +3.0 to +8.0 mm for the hippocampus.

3. Statistical Analysis

Data were presented as the means ± SEM. Statistical analysis of the results was performed by ANOVA, if the test prerequisites allowed, using SPSS Software v.29.0 (IBM Inc., Amonk, NY, USA). A two-way 2 (alcohol or saline) × 3 (Antalarmin or Astressin$_2$B or saline) ANOVA was performed with the estimated marginal means calculated followed by the Bonferroni post hoc test. A probability level of less than 0.05 was accepted as indicating a statistically significant difference.

4. Results

Hypothalamic CRF mRNA expression was increased by alcohol intoxication and withdrawal, and these stimulatory effects were reduced by antalarmin, but not astressin$_2$B (Figure 5). On the fifth day, a significant main effect in the alcohol-treated groups [$F(1,17) = 3.568$, $p < 0.001$], a significant main effect in the antagonist-treated group [$F(2,11) = 1.875$, $p < 0.001$], and a significant interaction between the two factors [$F(2,17) = 2.02$, $p < 0.001$] were observed. On the sixth day, a significant main effect in the alcohol-treated group [$F(1,17) = 12.476$, $p < 0.001$], a significant main effect in the antagonist-treated group [$F(2,11) = 8.535$, $p < 0.001$], and a significant interaction between the two factors [$F(2,17) = 17.435$, $p < 0.001$] were detected.

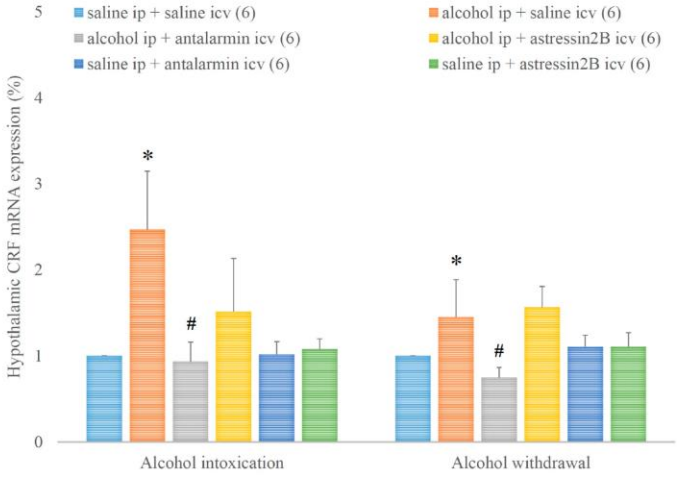

Figure 5. The effects of alcohol intoxication and withdrawal on the hypothalamic corticotropin-releasing factor (CRF) expression in rats and the impacts of antalarmin and astressin$_2$B on these effects. Values are presented as the means ± SEM; statistically significant difference was accepted for $p < 0.05$ and indicated with * for alcohol ip + saline icv vs. saline ip + saline icv and with # for alcohol ip + antalarmin icv vs. alcohol ip + saline icv.

In addition, the hypothalamic CRF concentration was increased by alcohol intoxication and withdrawal, and these stimulatory effects were reversed by the selective CRF1, but not CRF2 antagonist (Figure 6). On the 5th day, a significant main effect in the alcohol-treated group [$F(1,134) = 39.173$, $p < 0.001$], but no significant main effect in the antagonist-treated group, yet a significant interaction between the two factors [$F(1,17) = 3.356$, $p < 0.001$] were assessed. On the 6th day, a significant main effect in the alcohol-treated group [$F(1,35) = 10.021$, $p = 0.004$], but no significant main effect in the antagonist-treated group and no significant interaction between the two factors were noticed.

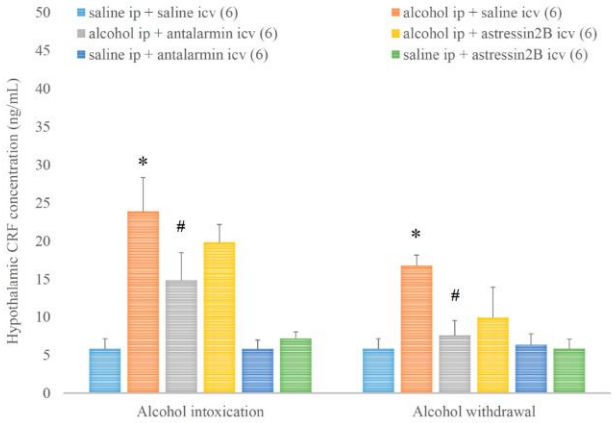

Figure 6. The effects of alcohol intoxication and withdrawal on the hypothalamic corticotropin-releasing factor (CRF) concentration in rats and the impacts of antalarmin and astressin$_2$B on these effects. Values are presented as the means ± SEM; statistically significant difference was accepted for $p < 0.05$ and indicated with * for alcohol ip + saline icv vs. saline ip + saline icv and with # for alcohol ip + antalarmin icv vs. alcohol ip + saline icv.

Hypothalamic AVP mRNA expression was decreased by alcohol intoxication and withdrawal, but these inhibitory effects were not significantly influenced either by antalarmin or astressin$_2$B (Figure 7). On the fifth day, a significant main effect in the alcohol-treated group [$F(1,17) = 3.451$, $p < 0.001$], no significant main effect in the antagonist-treated group, but a significant interaction between the two factors [$F(2,11) = 6.299$, $p < 0.001$] were observed. On the sixth day, a significant main effect in the alcohol-treated groups [$F(1,17) = 6.425$, $p < 0.001$], no significant main effect in the antagonist-treated group, but a significant interaction between the two factors [$F(2,11) = 10.245$, $p < 0.001$] were detected.

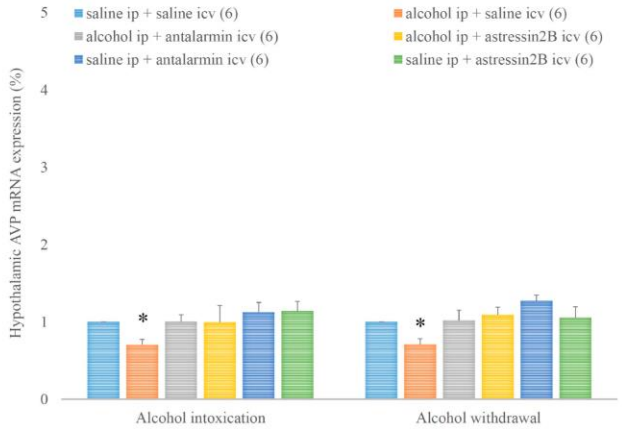

Figure 7. The effects of alcohol intoxication and withdrawal on the hypothalamic arginine vasopressin (AVP) expression in rats and the impacts of antalarmin and astressin$_2$B on these effects. Values are presented as the means ± SEM; statistically significant difference was accepted for $p < 0.05$ and indicated with * for alcohol ip + saline icv vs. saline ip + saline icv.

In contrast, hypothalamic AVP concentration was increased by alcohol intoxication and withdrawal, but these stimulatory effects were not affected by none of the selective CRF antagonists (Figure 8.). On the 5th day, a significant main effect in the alcohol-treated group [F(1,35) = 85.4, $p < 0.001$], but no significant main effect in the antagonist-treated group, and no significant interaction between the two factors were assessed. On the 6th day, a significant main effect in the alcohol-treated group [F(1,35) = 80.680, $p < 0.001$], but no significant main effect in the antagonist-treated group, and no significant interaction between the two factors were noticed.

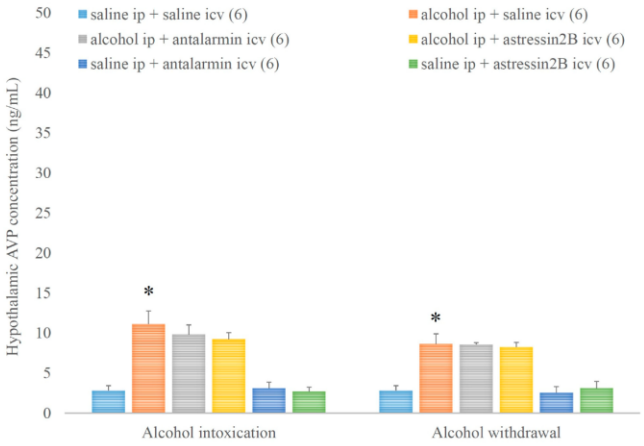

Figure 8. The effects of alcohol intoxication and withdrawal on the hypothalamic arginine vasopressin (AVP) concentration in rats and the impacts of antalarmin and astressin$_2$B on these effects. Values are presented as the means ± SEM; statistically significant difference was accepted for $p < 0.05$ and indicated with * for alcohol ip + saline icv vs. saline ip + saline icv.

The plasma ACTH level was elevated by alcohol intoxication and withdrawal, and these elevations were ameliorated by antalarmin, but not astressin$_2$B (Figure 9). On the fifth day, a significant main effect in the alcohol-treated group [F(1,35) = 64.352, $p < 0.001$], a significant main effect in the antagonist-treated group [F(2,35) = 12.523, $p < 0.001$], and a significant interaction between the two factors [F(2,35) = 9.311, $p < 0.001$] were proved. On the 6th day, a significant main effect in the alcohol-treated group [F(1,35) = 49.394, $p < 0.001$], but no significant main effect in the antagonist-treated group, and no significant interaction between the two factors were shown.

In parallel, the plasma CORT level was augmented by alcohol intoxication and withdrawal, and these augmentations were attenuated by the selective CRF1, but not CRF2 antagonist (Figure 10). On the fifth day, a significant main effect in the alcohol-treated group [F(1,35) = 30.996, $p < 0.001$], a significant main effect in the antagonist-treated group [F(2,35) = 3.892, $p < 0.001$], and a significant interaction between the two factors [F(2,35) = 4.778, $p < 0.001$] were shown. On the 6th day, a significant main effect in the alcohol-treated group [F(1,35) = 62.363, $p < 0.001$], a significant main effect in the antagonist-treated group [F(2,35) = 9.192, $p < 0.001$], and a significant interaction between the two factors [F(2,35) = 9.765, $p < 0.001$] were proved.

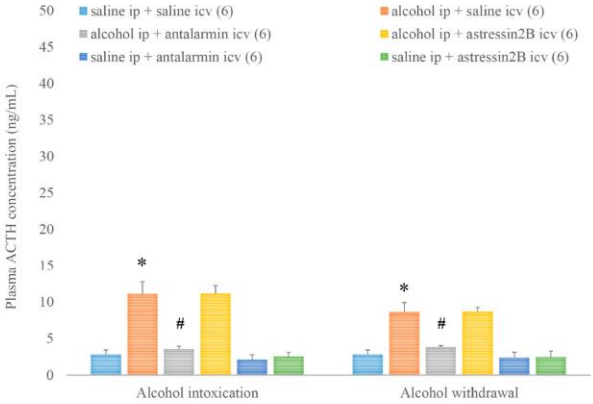

Figure 9. The effects of alcohol intoxication and withdrawal on the plasma adrenocorticoptropic hormone (ACTH) concentration in rats and the impacts of antalarmin and astressin$_2$B on these effects. Values are presented as the means ± SEM; statistically significant difference was accepted for $p < 0.05$ and indicated with * for alcohol ip + saline icv vs. saline ip + saline icv and with # for alcohol ip + antalarmin icv vs. alcohol ip + saline icv.

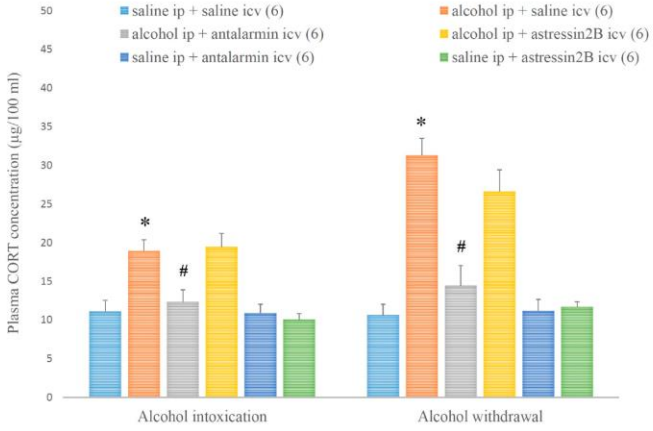

Figure 10. The effects of alcohol intoxication and withdrawal on the plasma corticosterone (CORT) concentration in rats and the impacts of antalarmin and astressin$_2$B on these effects. Values are presented as the means ± SEM; statistically significant difference was accepted for $p < 0.05$ and indicated with * for alcohol ip + saline icv vs. saline ip + saline icv and with # for alcohol ip + antalarmin icv vs. alcohol ip + saline icv.

Alcohol intoxication increased, whereas alcohol withdrawal decreased the striatal DA release, and both effects were reduced by antalarmin, but not astressin$_2$B (Figure 11). On the fifth day, a significant main effect in the alcohol-treated group [$F(1,17) = 271.724$, $p < 0.001$], a significant main effect in the antagonist-treated group [$F(2,11) = 98.352$, $p < 0.001$], and a significant interaction between the two factors [$F(2,11) = 100.708$, $p < 0.001$] were observed. On the sixth day, a significant main effect in the alcohol-treated group [$F(1,17) = 319.151$, $p < 0.001$], a significant main effect in the antagonist-treated group [$F(2,11) = 52.758$, $p < 0.001$], and a significant interaction between the two factors [$F(2,11) = 103.865$, $p < 0.001$] were detected.

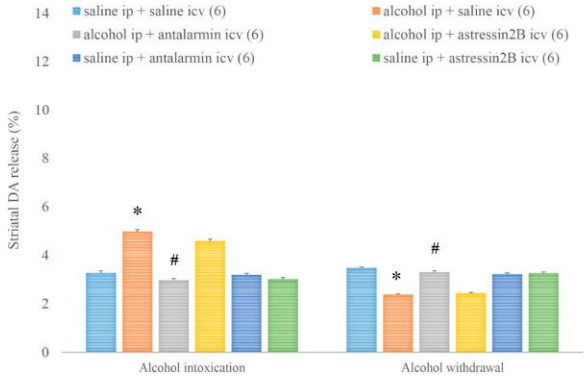

Figure 11. The effects of alcohol intoxication and withdrawal on striatal dopamine (DA) release in rats and the impacts of antalarmin and astressin$_2$B on these effects. Values are presented as means ± SEM; statistically significant difference was accepted for $p < 0.05$ and indicated with * for alcohol ip + saline icv vs. saline ip + saline icv and with # for alcohol ip + antalarmin icv vs. alcohol ip + saline icv.

Similarly, alcohol intoxication increased, whereas alcohol withdrawal decreased the amygdalar GABA release and both effects were reversed by the selective CRF1, but not CRF2 antagonist (Figure 12). On the fifth day, a significant main effect in the alcohol-treated group [$F(1,17) = 226.989$, $p < 0.001$], a significant main effect in the antagonist-treated group [$F(2,11) = 68.927$, $p < 0.001$], and a significant interaction between the two factors [$F(2,11) = 57.735$, $p < 0.001$] were assessed. On the sixth day, a significant main effect in the alcohol-treated group [$F(1,17) = 123.070$, $p < 0.001$], a significant main effect in the antagonist-treated group [$F(2,11) = 36.792$, $p < 0.001$], and a significant interaction between the two factors [$F(2,11) = 60.402$, $p < 0.001$] were noticed.

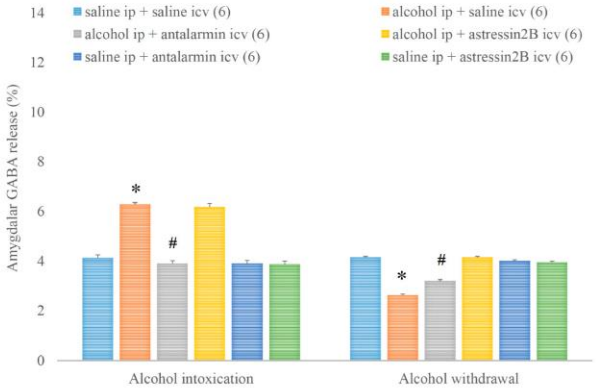

Figure 12. The effects of alcohol intoxication and withdrawal on the amygdalar gamma aminobutyric acid (GABA) release in rats and the impacts of antalarmin and astressin$_2$B on these effects. Values are presented as the means ± SEM; statistically significant difference was accepted for $p < 0.05$ and indicated with * for alcohol ip + saline icv vs. saline ip + saline icv and with # for alcohol ip + antalarmin icv vs. alcohol ip + saline icv.

Consequently, the hippocampal GLU release was decreased and increased in alcohol intoxication and alcohol withdrawal, respectively, and both effects were antagonized by antalarmin, but not astressin$_2$B (Figure 13). On the fifth day, a significant main effect in

the alcohol-treated group [F(1,17) = 663.538, $p < 0.001$], a significant main effect in the antagonist-treated group [F(2,11) = 172.546, $p < 0.001$], and a significant interaction between the two factors [F(2,11) = 257.706, $p < 0.001$] were shown. On the sixth day, a significant main effect in the alcohol-treated group [F(1,17) = 305.855, $p < 0.001$], a significant main effect in the antagonist-treated group [F(2,11) = 97.827, $p < 0.001$], and a significant interaction between the two factors [F(2,11) = 82.538, $p < 0.001$] were proved.

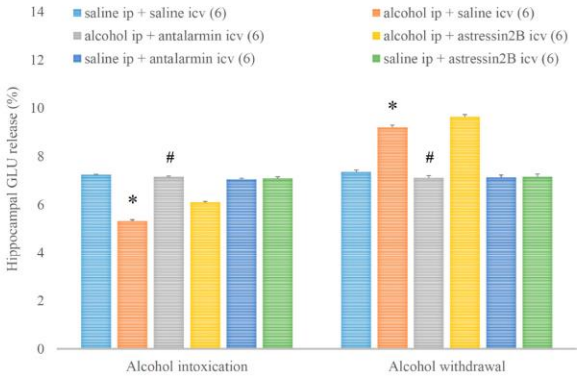

Figure 13. The effects of alcohol intoxication and withdrawal on the hippocampal glutamate (GLU) release in rats and the impacts of antalarmin and astressin$_2$B on these effects. Values are presented as the means ± SEM; statistically significant difference was accepted for $p < 0.05$ and indicated with * for alcohol ip + saline icv vs. saline ip + saline icv and with # for alcohol ip + antalarmin icv vs. alcohol ip + saline icv.

5. Discussion

Our results indicate that during alcohol intoxication, the HPA axis is activated by hypothalamic CRF and is reflected by the elevation of plasma CORT and ACTH levels. Aside from the stress axis, the reward system is also activated, mainly resulting in increased striatal DA, but also increased amygdalar GABA and decreased hippocampal GLU release. During alcohol withdrawal, the HPA axis remains activated, but this time, the activation is accompanied by the decrease in the striatal DA and amygdalar GABA release, and increase in hippocampal GLU release, which is probably mediated by extrahypothalamic CRF.

Regarding the hypothalamic neurohormones, previous studies have already suggested the role of CRF in the HPA-axis activation induced by alcohol based on several lines of evidence [21]. First, the administration of a CRF antiserum or a CRF antagonist inhibited the stimulatory effect of alcohol on ACTH secretion in rats [22–24]. Second, bilateral destruction of the paraventricular CRF-secreting neurons ameliorated, although did not abolish, the alcohol-stimulated ACTH secretion [25,26]. Third, the administration of alcohol increased the expression of CRF heteronuclear RNA and mRNA levels as well as the expression of c-Fos mRNA and the Fos protein in the PVN [25,27,28]. Furthermore, the present study emphasizes the role of hypothalamic CRF in the activation of the HPA axis during alcohol intoxication and withdrawal. This finding is supported by the observation that both the expression and concentration of the hypothalamic CRF increased in parallel with the levels of plasma ACTH and CORT, immediately and 24 h after the last alcohol administration. Previous studies have also suggested the role of AVP in the alcohol-induced activation of the HPA-axis [21]. First, the administration of an AVP antiserum or an AVP antagonist inhibited the stimulatory effect of alcohol on ACTH secretion in rats [24,29]. Second, the removal of endogenous AVP in rats previously exposed to bilateral destruction of the paraventricular neurons partially diminished the alcohol-stimulated ACTH secretion [25]. Third, the administration of alcohol increased the expression of AVP heteronuclear RNA and mRNA levels [27]. However, the present study questions the role of the hypothalamic

AVP in activating the HPA axis during alcohol intoxication and withdrawal. This is based on the speculation that the decreased hypothalamic AVP expression and the increased hypothalamic AVP concentration immediately and 24 h after the last alcohol administration represents a decreased release rather than an increased synthesis of hypothalamic AVP, a process that might have resulted from the negative feedback of plasma glucocorticoids and ACTH, or could be related to another function of AVP such as water retention.

Regarding the extrahypothalamic neurotransmitters, previous studies have suggested that alcohol binge/intoxication and withdrawal/negative effects are also associated with certain changes in the striatal DA, amygdalar GABA, and hippocampal GLU [1,2]. Acute alcohol administration stimulates the striatal DA release, inducing the sensation of reward, whereas chronic alcohol administration leads to a decrease in the DA release in the striatum, which is manifested in a reward deficit during alcohol withdrawal [30,31]. This reward deficit can be explained by an increase in the reward threshold caused by the downregulation of pre-synaptic DA receptors, and a decrease in extracellular DA release caused by the depletion of striatal DA stores that are demasked during alcohol withdrawal [32]. The amygdalar GABA is presumed to play a role in the positive, anxiolytic effects of alcohol [33–36]. Acute alcohol consumption facilitates GABA-ergic neurotransmission in CeA via both pre- and post-synaptic mechanisms, whereas chronic alcohol consumption increases the baseline GABA-ergic neurotransmission, but not the stimulated GABA release [37]. The hippocampal GLU is believed to play a role in the negative, anxiogenic effects of alcohol and the development of aggression observed especially during alcohol withdrawal [12,38–40]. In general, acute alcohol consumption decreases glutamatergic neurotransmission by the downregulation of N-methyl-d-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, whereas chronic alcohol consumption increases glutamatergic neurotransmission by the upregulation of NMDA receptors and the stimulation of GLU release, which might be further enhanced by repeated periods of alcohol withdrawal [37].

As for the role of CRF receptors in the neurohormonal changes induced by alcohol, previous in vivo experiments have already reported that alcohol induced upregulation of paraventricular CRF1 expression [41], and that the alcohol-induced ACTH secretion could be blocked by the non-selective CRF receptor antagonist astressin and selective CRF1 antagonist NBI 30775 [42]. In contrast, alcohol administration was unable to produce the upregulation of CRF2 expression in the PVN [41], and the alcohol-induced ACTH secretion could not be blocked by the selective CRF2 antagonist astressin$_2$B [42]. This is in agreement with our previous in vitro experiments that demonstrated that striatal DA and amygdalar GABA release could be stimulated or inhibited by non-selective CRF1 agonists or antagonists, but are not affected by selective CRF2 agonists or antagonists [43–45]. In accordance with the previous findings, our present study underlines the role of CRF1 in the changes in hypothalamic neurohormones and extrahypothalamic neurotransmitters observed during alcohol intoxication and withdrawal. Nevertheless, based on the present findings, the role of CRF receptors in the alcohol-induced alteration of the hypothalamic AVP can be excluded.

6. Conclusions

In conclusion, our results indicate that the neuroendocrine changes induced by alcohol intoxication and withdrawal are mediated by CRF1, not CRF2, except for the changes in hypothalamic AVP, which are not mediated by CRF receptors. Therefore, our study demonstrates that 4-day ip administration of alcohol followed by 1-day abstinence in rats is a valid model for alcohol intoxication and withdrawal, characterized by specific changes in hypothalamic neurohormones and extrahypothalamic neurotransmitters, which can be used for therapeutical purposes.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets generated during the current study are available from the corresponding author upon reasonable request.

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References

1. Bruijnzeel, A.W.; Gold, M.S. The role of corticotropin-releasing factor-like peptides in cannabis, nicotine, and alcohol dependence. *Brain Res. Rev.* **2005**, *49*, 505–528. [CrossRef] [PubMed]
2. Sarnyai, Z.; Shaham, Y.; Heinrichs, S.C. The role of corticotropin-releasing factor in drug addiction. *Pharmacol. Rev.* **2001**, *53*, 209–243.
3. Koob, G.F.; Volkow, N.D. Neurocircuitry of addiction. *Neuropsychopharmacology* **2010**, *35*, 217–238. [CrossRef] [PubMed]
4. Carrasco, G.A.; Van de Kar, L.D. Neuroendocrine pharmacology of stress. *Eur. J. Pharmacol.* **2003**, *463*, 235–272. [CrossRef]
5. Tsigos, C.; Chrousos, G.P. Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. *J. Psychosom. Res.* **2002**, *53*, 865–871. [CrossRef]
6. Koob, G.F. The role of CRF and CRF-related peptides in the dark side of addiction. *Brain Res.* **2010**, *1314*, 3–14. [CrossRef]
7. Van Pett, K.; Viau, V.; Bittencourt, J.C.; Chan, R.K.; Li, H.Y.; Arias, C.; Prins, G.S.; Perrin, M.; Vale, W.; Sawchenko, P.E. Distribution of mRNAs encoding CRF receptors in brain and pituitary of rat and mouse. *J. Comp. Neurol.* **2000**, *428*, 191–212. [CrossRef]
8. Vale, W.; Spiess, J.; Rivier, C.; Rivier, J. Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. *Science* **1981**, *213*, 1394–1397. [CrossRef]
9. Bale, T.L.; Lee, K.F.; Vale, W.W. The role of corticotropin-releasing factor receptors in stress and anxiety. *Integr. Comp. Biol.* **2002**, *42*, 552–555. [CrossRef]
10. Bale, T.L.; Vale, W.W. CRF and CRF receptors: Role in stress responsivity and other behaviors. *Annu. Rev. Pharmacol. Toxicol.* **2004**, *44*, 525–557. [CrossRef]
11. Paxinos, G.; Watson, C. *The Rat Brain in Stereotaxic Coordinates*, 6th ed.; Academic Press/Elsevier: Amsterdam, The Netherlands; Boston, MA, USA, 2007.
12. Silvestre de Ferron, B.; Bennouar, K.E.; Kervern, M.; Alaux-Cantin, S.; Robert, A.; Rabiant, K.; Antol, J.; Naassila, M.; Pierrefiche, O. Two Binges of Ethanol a Day Keep the Memory Away in Adolescent Rats: Key Role for GLUN2B Subunit. *Int. J. Neuropsychopharmacol.* **2015**, *19*, pyv087. [CrossRef] [PubMed]
13. Buzas, A.; Bokor, P.; Balango, B.; Pinter, D.; Palotai, M.; Simon, B.; Csabafi, K.; Telegdy, G.; Szabo, G.; Bagosi, Z. Changes in striatal dopamine release and locomotor activity following acute withdrawal from chronic nicotine are mediated by CRF1, but not CRF2, receptors. *Brain Res.* **2019**, *1706*, 41–47. [CrossRef] [PubMed]
14. Bagosi, Z.; Palotai, M.; Simon, B.; Bokor, P.; Buzas, A.; Balango, B.; Pinter, D.; Jaszberenyi, M.; Csabafi, K.; Szabo, G. Selective CRF2 receptor agonists ameliorate the anxiety- and depression-like state developed during chronic nicotine treatment and consequent acute withdrawal in mice. *Brain Res.* **2016**, *1652*, 21–29. [CrossRef]
15. Suda, T.; Yajima, F.; Tomori, N.; Demura, H.; Shizume, K. In vitro study of immunoreactive corticotropin-releasing factor release from the rat hypothalamus. *Life Sci.* **1985**, *37*, 1499–1505. [CrossRef] [PubMed]
16. Joanny, P.; Steinberg, J.; Zamora, A.J.; Conte-Devolx, B.; Millet, Y.; Oliver, C. Corticotropin-releasing factor release from in vitro superfused and incubated rat hypothalamus. Effect of potassium, norepinephrine, and dopamine. *Peptides* **1989**, *10*, 903–911. [CrossRef]
17. Purves, H.D.; Sirett, N.E. Assay of corticotrophin in dexamethasone-treated rats. *Endocrinology* **1965**, *77*, 366–374. [CrossRef]
18. Zenker, N.; Bernstein, D.E. The estimation of small amounts of corticosterone in rat plasma. *J. Biol. Chem.* **1958**, *231*, 695–701. [CrossRef]
19. Gaddum, J.H. The technique of superfusion. *Br. J. Pharmacol. Chemother.* **1953**, *8*, 321–326. [CrossRef]

20. Harsing, L.G., Jr.; Vizi, E.S. Release of endogenous dopamine from rat isolated striatum: Effect of clorgyline and (-)-deprenyl. *Br. J. Pharmacol.* **1984**, *83*, 741–749. [CrossRef]
21. Rivier, C. Role of hypothalamic corticotropin-releasing factor in mediating alcohol-induced activation of the rat hypothalamic-pituitary-adrenal axis. *Front. Neuroendocrinol.* **2014**, *35*, 221–233. [CrossRef]
22. Rivier, C.; Bruhn, T.; Vale, W. Effect of ethanol on the hypothalamic-pituitary-adrenal axis in the rat: Role of corticotropin-releasing factor (CRF). *J. Pharmacol. Exp. Ther.* **1984**, *229*, 127–131. [PubMed]
23. Rivier, C.; Rivier, J.; Vale, W. Inhibition of adrenocorticotropic hormone secretion in the rat by immunoneutralization of corticotropin-releasing factor. *Science* **1982**, *218*, 377–379. [CrossRef]
24. Rivier, C.; Vale, W. Modulation of stress-induced ACTH release by corticotropin-releasing factor, catecholamines and vasopressin. *Nature* **1983**, *305*, 325–327. [CrossRef] [PubMed]
25. Ogilvie, K.M.; Lee, S.; Rivier, C. Role of arginine vasopressin and corticotropin-releasing factor in mediating alcohol-induced adrenocorticotropin and vasopressin secretion in male rats bearing lesions of the paraventricular nuclei. *Brain Res.* **1997**, *744*, 83–95. [CrossRef] [PubMed]
26. Rivest, S.; Rivier, C. Lesions of hypothalamic PVN partially attenuate stimulatory action of alcohol on ACTH secretion in rats. *Am. J. Physiol.* **1994**, *266*, R553–R558. [CrossRef] [PubMed]
27. Rivier, C.; Lee, S. Acute alcohol administration stimulates the activity of hypothalamic neurons that express corticotropin-releasing factor and vasopressin. *Brain Res.* **1996**, *726*, 1–10. [CrossRef]
28. Ogilvie, K.; Lee, S.; Rivier, C. Effect of three different modes of alcohol administration on the activity of the rat hypothalamic-pituitary-adrenal axis. *Alcohol. Clin. Exp. Res.* **1997**, *21*, 467–476. [CrossRef]
29. Rivier, C. Gender, sex steroids, corticotropin-releasing factor, nitric oxide, and the HPA response to stress. *Pharmacol. Biochem. Behav.* **1999**, *64*, 739–751. [CrossRef]
30. Budygin, E.A.; John, C.E.; Mateo, Y.; Daunais, J.B.; Friedman, D.P.; Grant, K.A.; Jones, S.R. Chronic ethanol exposure alters presynaptic dopamine function in the striatum of monkeys: A preliminary study. *Synapse* **2003**, *50*, 266–268. [CrossRef]
31. Budygin, E.A.; Phillips, P.E.; Robinson, D.L.; Kennedy, A.P.; Gainetdinov, R.R.; Wightman, R.M. Effect of acute ethanol on striatal dopamine neurotransmission in ambulatory rats. *J. Pharmacol. Exp. Ther.* **2001**, *297*, 27–34.
32. Vengeliene, V.; Bilbao, A.; Molander, A.; Spanagel, R. Neuropharmacology of alcohol addiction. *Br. J. Pharmacol.* **2008**, *154*, 299–315. [CrossRef]
33. Herman, M.A.; Kallupi, M.; Luu, G.; Oleata, C.S.; Heilig, M.; Koob, G.F.; Ciccocioppo, R.; Roberto, M. Enhanced GABAergic transmission in the central nucleus of the amygdala of genetically selected Marchigian Sardinian rats: Alcohol and CRF effects. *Neuropharmacology* **2013**, *67*, 337–348. [CrossRef]
34. McBride, W.J. Central nucleus of the amygdala and the effects of alcohol and alcohol-drinking behavior in rodents. *Pharmacol. Biochem. Behav.* **2002**, *71*, 509–515. [CrossRef]
35. Diaz, M.R.; Morton, R.A. Ethanol untangles the amygdala-anxiety circuit through tonic GABA inhibition. *Alcohol. Clin. Exp. Res.* **2014**, *38*, 619–623. [CrossRef]
36. Roberto, M.; Madamba, S.G.; Stouffer, D.G.; Parsons, L.H.; Siggins, G.R. Increased GABA release in the central amygdala of ethanol-dependent rats. *J. Neurosci.* **2004**, *24*, 10159–10166. [CrossRef]
37. Roberto, M.; Gilpin, N.W.; Siggins, G.R. The central amygdala and alcohol: Role of gamma-aminobutyric acid, glutamate, and neuropeptides. *Cold Spring Harb. Perspect. Med.* **2012**, *2*, a012195. [CrossRef]
38. Chefer, V.; Meis, J.; Wang, G.; Kuzmin, A.; Bakalkin, G.; Shippenberg, T. Repeated exposure to moderate doses of ethanol augments hippocampal glutamate neurotransmission by increasing release. *Addict. Biol.* **2011**, *16*, 229–237. [CrossRef]
39. Martin, D.; Swartzwelder, H.S. Ethanol inhibits release of excitatory amino acids from slices of hippocampal area CA1. *Eur. J. Pharmacol.* **1992**, *219*, 469–472. [CrossRef]
40. Sepulveda, C.; Bustos, G.; Gysling, K.; Seguel, M.; Labarca, R. Effects of in vitro ethanol and chronic ethanol consumption on the release of excitatory amino acids in the rat hippocampus. *Brain Res.* **1995**, *674*, 104–106. [CrossRef]
41. Lee, S.; Rivier, C. Alcohol increases the expression of type 1, but not type 2 alpha corticotropin-releasing factor (CRF) receptor messenger ribonucleic acid in the rat hypothalamus. *Brain Res. Mol. Brain Res.* **1997**, *52*, 78–89. [CrossRef]
42. Rivier, C.L.; Grigoriadis, D.E.; Rivier, J.E. Role of corticotropin-releasing factor receptors type 1 and 2 in modulating the rat adrenocorticotropin response to stressors. *Endocrinology* **2003**, *144*, 2396–2403. [CrossRef]
43. Bagosi, Z.; Jaszberenyi, M.; Bujdoso, E.; Telegdy, G. The effects of corticoptropin-releasing factor and the urocortins on striatal dopamine release induced by electrical stimulation-an in vitro superfusion study. *Neurochem. Res.* **2006**, *31*, 209–213. [CrossRef]
44. Bagosi, Z.; Jaszberenyi, M.; Szabo, G.; Telegdy, G. The effects of CRF and the urocortins on [3H]GABA release from the rat amygdala—An in vitro superfusion study. *Brain Res. Bull.* **2008**, *75*, 15–17. [CrossRef]
45. Bagosi, Z.; Balango, B.; Pinter, D.; Csabafi, K.; Jaszberenyi, M.; Szabo, G.; Telegdy, G. The effects of CRF and urocortins on the hippocampal glutamate release. *Neurochem. Int.* **2015**, *90*, 67–71. [CrossRef]

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The effects of alcohol on anxiety-like, depression-like, and social behavior immediately and a day after binge drinking

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ABSTRACT

The aim of the present study was to determine the effects of binge drinking on anxiety-like, depression-like, and social behavior. The participation of the corticotropin-releasing factor (CRF) receptors (CRF1 and CRF2) in these effects was also investigated. Therefore, male C57BL/6 mice were exposed to drinking in the dark, a classical animal model for binge drinking, and treated intracerebroventricularly (icv) with selective CRF1 antagonist antalarmin or selective CRF2 antagonist astressin$_2$B, immediately or 24 h after binge drinking. After 30 min, the animals were investigated in an elevated plus-maze test and a forced swim test for anxiety-like and depression-like signs, respectively. In addition, mice were tested in a three-chamber social interaction arena for sociability and preference for social novelty. Immediately after binge drinking, mice exposed to alcohol expressed anxiolytic and antidepressant effects, which were reduced by astressin$_2$B, but not antalarmin. Moreover, mice exposed to alcohol showed increased sociability and preference for social novelty immediately after binge drinking. In contrast, 24 h after binge drinking mice exposed to alcohol presented anxiety-like and depression-like signs, which were reversed by antalarmin, but not astressin$_2$B. However, mice exposed to alcohol did not show any significant change in social interaction after 24 h. The present study demonstrates that alcohol exerts different effects on anxiety-like, depression-like, and social behavior immediately and a day after binge drinking, and that the anxiolytic and antidepressant effects produced by binge drinking are mediated by CRF2, whereas the anxiety-like and depression-like signs observed the next day are promoted by CRF1.

Introduction

Binge drinking is defined as consuming a large amount of alcohol in a short period of time (Chung, Creswell, Bachrach, Clark, & Martin, 2018). A large amount of alcohol refers to five or more alcoholic drinks in men and four or more alcoholic drinks in women that, by definition, brings their blood alcohol concentration (BAC) to 0.08 g/dL within 2 h, and is commonly associated with acute impairment in motor coordination and cognitive functioning (Chung et al., 2018). Hangover is a temporary state described as the unpleasant next-day effects after binge drinking (van Schrojenstein Lantman, van de Loo, Mackus, & Verster, 2016). This state usually emerges after a single episode of heavy drinking when BAC approaches zero and is associated with a combination of physical signs, such as ataxia, locomotor and exploratory dysfunctions, and affective symptoms, such as fear, anxiety, and depression (van Schrojenstein Lantman et al., 2016). Alcoholism is best resembled by alternating episodes of binge drinking and hangover (Koob, 2013, 2014). Individuals who regularly engage in episodic heavy drinking do not entirely meet the diagnostic criteria for alcoholism; however, repeated cycles of binge drinking that emerge during adolescence are an important risk factor for development of alcohol addiction in adulthood (Koob, 2013, 2014). Furthermore, repeated episodes of binge drinking may elicit persistent negative affect, including anxiety and depression (Jimenez Chavez et al., 2022; Lee, Coehlo, McGregor, Waltermire, & Szumlinski, 2015; Lee, Coehlo, Solton, & Szumlinski, 2017; Olney, Marshall, & Thiele, 2018), and alteration of social behavior

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similar to that described during acute alcohol withdrawal (Kent, Butler, & Wood, 2014; Wood, Knoll, & Levitt, 2015). Nevertheless, anxiety, depression, and hangover are usually attributed to humans. In the present study we used male C57BL/6 mice, hereby we refer to these terms as anxiety-like and depression-like signs, and hangover-like symptoms.

The aim of the present study was to determine the effects of binge drinking on anxiety-like, depression-like, and social behavior. For this purpose, male C57BL/6 mice were exposed to drinking in the dark, a classic method to investigate binge drinking in animals (Rhodes, Best, Belknap, Finn, & Crabbe, 2005). Corticotropin-releasing factor (CRF) and its receptors (CRF1 and CRF2) have been involved in the pathogenesis of anxiety and depression (Reul & Holsboer, 2002), and various aspects of social behavior (Bagosi, Czébely-Lénárt et al., 2017; Bagosi, Karasz, et al., 2017). Therefore, the participation of the CRF receptors (CRF1 and CRF2) in these effects was also investigated. In order to do so, first the dark–light cycle of the mice was inverted for 14 days and then their water bottles were replaced by bottles of 20% alcohol for 4 days (2 h on the first, second, and third day, and 4 h on the fourth day). On the 4th day, immediately after binge drinking, or on the 5th day, 24 h after binge drinking, mice were treated intracerebroventricularly (icv) with selective CRF1 antagonist antalarmin or selective CRF2 antagonist astressin$_2$B. After 30 min, the animals were investigated in an elevated plus-maze test and a forced swim test for signs of anxiety and depression, respectively. In addition, mice were tested in a three-chamber social interaction arena for sociability and preference for social novelty.

Materials and methods

Animals

Male C57BL/6 mice (Charles River Laboratories Ltd., Hungary) 6 weeks old, weighing 18–24 g, were used. The mice were kept in their home cages at a constant temperature on a standard illumination schedule with 12-h light and 12-h dark periods (lights on from 6:00 PM to 6:00 AM). Commercial food and tap water were available *ad libitum*. To minimize the effects of non-specific stress the mice were handled daily. All tests were performed between 9:00 AM to 12:00 noon. The animals were treated in accordance with the ARRIVE guidelines and the experiments were carried out in accordance with the EU Directive 2010/63/EU for animal experiments.

Surgery

The mice were implanted with a stainless-steel Luer cannula, aimed at the right lateral cerebral ventricle under anesthesia with 60 mg/kg pentobarbital sodium (Euthanasol, CEVA-Phylaxia Ltd., Hungary). The stereotaxic coordinates were 0.5 mm lateral and 0.5 mm posterior from the bregma and 3 mm deep from the dural surface according to the stereotaxic atlas of the mouse brain (Paxinos & Franklin, 2004). Cannulas were secured to the skull with Ferrobond instant glue (Ferrokémia Ltd., Hungary) and they were closed by a metal string between injections. Before the experiments, the mice were allowed to recover for 5 days after the surgery. After the experiments, 4 μL of dye methylene blue (Reanal Ltd., Hungary) at 1 g/100 mL concentration was injected through the cannula to identify the site of injection. Animals without the dye in the lateral cerebral ventricle were discarded.

Drinking in the dark

The mice were exposed to drinking in the dark, a classic animal model for binge drinking (Rhodes et al., 2005). First, *the* dark–light cycle of the mice was inverted for 14 days, and then their water bottles were replaced by bottles of 20% alcohol (Reanal Ltd., Hungary) for 4 days (2 h on the first, second, and third day, and 4 h on the fourth day).

Treatment

On the 4th day (immediately after binge drinking) or on the 5th day (24 h after binge drinking) mice were treated icv with the selective CRF1 antagonist antalarmin (Sigma–Aldrich Ltd., Hungary) or the selective CRF2 antagonist astressin$_2$B (Sigma–Aldrich Ltd., Hungary). The mice were assigned randomly for one of the treatments on the 4th day or the 5th day; they were not exposed to more than one icv administration in two consecutive days. The dose of antalarmin was 0.1 μg/2 μL, and that of astressin$_2$B was 1 μg/2 μL. As in our previous experiments these doses have been already been proven to effectively block the neuroendocrine stress response, without altering the social behavior of animals (Bagosi, Czebely-Lenart et al., 2017; Bagosi, Karasz, et al., 2017). After 30 min the animals were investigated in an elevated plus-maze test or a forced swim test for signs of anxiety or depression, respectively. In parallel, a three-chamber social interaction test was also performed, in order to investigate the sociability and the preference for social novelty of mice. Also, the mice were assigned randomly for one of the behavioral tests; they were not exposed consequently to the elevated plus-maze, forced swim, or social interaction test.

Elevated plus-maze test

The mice were investigated in an elevated plus-maze test described first by Lister (1987). The elevated plus-maze apparatus consists of a plus-shaped wooden platform elevated at 40 cm from the floor, made up of four opposing arms of 30 cm × 5 cm. Two of the opposing arms are enclosed by 15-cm high side and end walls (closed arms), whereas the other two arms have no walls (open arms). The room where the behavioral tests were performed had been darkened, with only the central area of the elevated plus-maze illuminated with a lamp 50 cm from the platform, having an LED bulb of 3.5 W that produces 2230 lumens. The principle of the test is that open arms are more fear-provoking than the closed arms, and the ratio of the time spent in open vs. closed arms, or the ratio of the entries into open vs. closed arms, reflects the relative safety of closed arms, as compared with the relative danger of open arms. Each mouse was placed in the central area of 5 cm × 5 cm of the maze, facing one of the open arms. For a 5-min period, two parameters were recorded by an observer sitting 100 cm from the center of the plus-maze: 1) the number of entries into the open arms relative to the total number of entries, and 2) the time spent in the open arms relative to the total time. All parameters were expressed as percentages. The platform of the apparatus was cleaned with sodium hypochlorite solution (HIP-TOM Ltd., Hungary) between the subjects.

Forced swim test

The mice were also investigated in a forced swim test described first by Porsolt and co-workers (Porsolt, Bertin, & Jalfre, 1977). The forced swim apparatus consists of a plexiglass cylinder of 40-cm height and 12-cm diameter positioned on a table. The cylinder

was half-filled with water maintained at 25 ± 1 °C. The principle of the test is that in such a situation, from which they cannot escape, animals rapidly became immobile, that is, floating in an upright position and making only small movements to keep their heads above water. Meanwhile, their attempts to escape the cylinder by climbing or swimming may decrease or cease eventually. Each mouse was placed individually into the water. For a 5-min period the following parameters were recorded by an observer sitting 100 cm from the table: the time that mice spent with swimming and climbing the walls, in their attempt to remain at the surface and escape the cylinder, respectively, and the time spent with immobility or floating. All parameters were expressed in time units, a time unit consisting of 5 s. The water from the cylinder was completely changed between the subjects.

Three-chamber social interaction test

The mice were also tested in a social interaction arena invented by Crawley and co-workers (Crawley et al., 2007). The arena is represented by a rectangular plexiglass box divided into three chambers, each chamber having the dimensions of 19 × 45 × 25 cm. The right and left chambers could be isolated from the middle one by using two dividing plexiglass walls. Two identical, wire cup-like cages of 10 × 17 cm with removable lids large enough to hold a single mouse were placed vertically inside the apparatus, one in each side chamber. Each cage was composed of metal wires to allow for air exchange between the interior and exterior of the cylinder but was small enough to prevent direct physical (aggressive or sexual) interactions between the animal on the inside with the animal on the outside. Two types of tests were performed: the first test was meant to measure the sociability, whereas the second test was meant to measure the preference for social novelty of the mice (Kaidanovich-Beilin, Lipina, Vukobradovic, Roder, & Woodgett, 2011). In the first test, the tested mouse was first habituated with the middle chamber for 5 min and then allowed to explore the remaining chambers for another 5 min. Then, a stranger male mouse in a cage was placed into one of the chambers and an empty cage was put into the other chamber. The principle of the first test is based on the observation that a wild-type mouse enters and spends more time in interaction with the stranger mouse over a foreign object (e.g., empty cage), indicative of intact sociability. In the second test, a stranger male mouse in a cage was placed into one of the chambers and the male mouse that was the stranger in the previous test (now considered familiarized) in a cage was placed into the opposite chamber. The principle of this second test is based on the assessment that a wild-type mouse enters and spends more time in interaction with the stranger mouse over the familiarized one, indicating a natural preference for social novelty. In both tests the following parameters were recorded by an observer sitting 200 cm from the box for two 5-min periods: the number of entries into the chamber relative to the total number of entries, and the time of interaction with the stranger relative to the total time of interaction. The number of entries was counted when both the head and the four paws of the tested mouse had entered into the chamber. The time of interaction was measured when the tested mouse was at least 3 cm from the cage. The floors and the walls of the arena were cleaned with sodium hypochlorite solution (HIP-TOM Ltd., Hungary) between the tests.

Blood alcohol concentration (BAC) measurement

In our study the amount of alcohol consumed was calculated by bottle weight each day, and BAC was determined only on the 4th day (immediately after binge drinking) and on the 5th day (24 h after binge drinking) for each mouse. The mice were decapitated, and trunk blood was collected after the behavioral tests. Ethanol was determined from the plasma obtained by centrifugation of the trunk blood, immediately after sample collection by commercially available enzymatic kit (Ref. No. 03183777 190, Roche Diagnostics, Mannheim, Germany) on a cobas c502 analyzer (Roche Diagnostics). The sensitivity of the assay was 10.1 mg/dL (0.01 g/dL). Based on previous experiments, drinking in the dark results in alcohol intakes between 3.5 and 5.0 g/kg alcohol (Thiele, Crabbe, & Boehm, 2014; Thiele & Navarro, 2014). The intake of this amount of alcohol should produce a BAC of 0.08 g/dL within 2 h in C57BL/6 mice (Thiele et al., 2014; Thiele & Navarro, 2014). However, in the present experiments, mice did not always reach the alcohol level that is characteristic for binge drinking; therefore, mice with BAC lower than 0.08 g/dL were excluded from the statistical analysis.

Statistical analysis

Statistical analysis of the results was performed by analysis of variance (GraphPad Prism, GraphPad Software Inc., United States). The differences between groups were determined by one-way ANOVA, followed by Tukey's *post hoc* test for pair-wise comparisons. The probability level of 0.05 or less was accepted as indicating a statistically significant difference.

Results

On the 4th day (immediately after binge drinking), the number of entries into and the time spent in the open arms of the elevated plus-maze increased significantly in mice exposed to alcohol, compared to the control mice (Fig. 1). Consequently, mice exposed to alcohol spent significantly more time with swimming and climbing, and significantly less time with floating in the water, when compared to the control mice (Fig. 2). These anxiolytic and antidepressant effects of alcohol were decreased significantly by astressin$_2$B, but not the antalarmin (Figs. 1 and 2). The number of entries to the stranger was not affected in the social interaction tests, but mice exposed to alcohol spent significantly more time with the stranger in both social interaction tests, when compared to the control (Figs. 3 and 4). These signs of enhanced sociability and preference for social novelty were reduced significantly by antalarmin, but not astressin$_2$B (Figs. 3 and 4). The results of the statistical analysis were summarized in a separate table for each test (Tables 1–4). The results of the BAC measurements were also summarized in a table (Table 5). The BACs of the mice exposed to binge drinking were 0.10 ± 0.02 g/dL.

On the 5th day (24 h after binge drinking), the number of entries into and the time spent in the open arms were decreased significantly in mice exposed previously to alcohol, compared to the control (Fig. 1). Also, mice exposed previously to alcohol spent significantly less time with swimming and climbing, and significantly more time with floating in the water, when compared to the control mice (Fig. 2). These signs of anxiety and depression were reversed significantly by antalarmin, but not astressin$_2$B (Figs. 1 and 2). However, previous exposure to alcohol did not affect the number of entries to or the time spent with the stranger in either of the social interaction tests (Figs. 3 and 4). Accordingly, neither antalarmin nor astressin$_2$B did influence considerably the sociability and preference for social novelty of the mice (Figs. 3 and 4). The results of the statistical analysis were summarized in a separate table for each test (Tables 1–4). The results of the BAC measurements were also summarized in a table (Table 5). As we previously mentioned, mice did not always reach the alcohol level that is characteristic for binge drinking; therefore, mice with BACs lower than 0.08 g/dL were excluded from the statistical analysis.

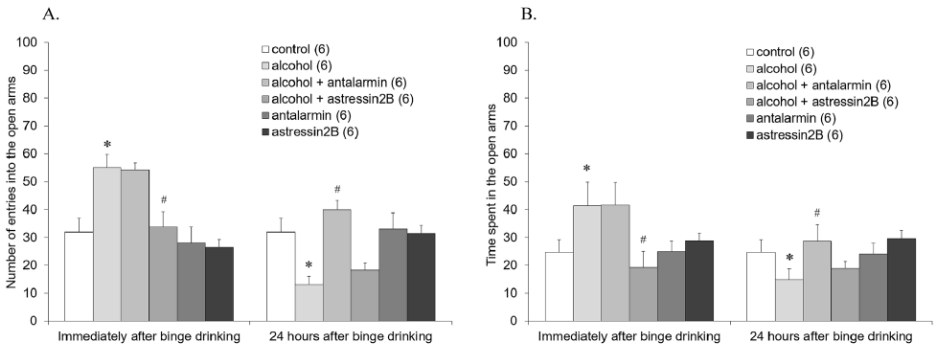

Fig. 1. The effects of binge drinking on the number of entries into (**A**), and the time spent in the open arms (**B**) in mice investigated in an elevated plus-maze test for signs of anxiety. Values are presented as means ± SEM; a statistically significant difference was accepted for $p < 0.05$ and indicated with * for alcohol vs. control, and # for alcohol + CRF antagonist vs. alcohol alone.

Discussion

The present study demonstrates that alcohol exerts different effects on anxiety-like, depression-like, and social behavior immediately and a day after binge drinking. Binge drinking produces anxiolytic and antidepressant effects when mice are tested immediately after drinking in the dark. Previous studies have already suggested that a single cycle of binge drinking is not necessarily associated with anxiety and depression (Evans, Rodríguez-Borillo, Font, Currie, & Pastor, 2020; Olney et al., 2018). In concordance, a recent study using a slightly modified version of the drinking in the dark paradigm showed that binge drinking has no short-term effect on the behavior of adolescent C57BL/6 mice but evokes anxiety- and depressive-like behavior during adulthood (Van Hees et al., 2022). Based on the present experiments, a single session of binge drinking in adolescent C57BL/6 mice seems to have rather anxiolytic and antidepressant effects. In addition, binge drinking enhances the sociability and the preference for social novelty of male mice when they are tested immediately after drinking in the dark, a finding that can be related to the anxiolytic and antidepressant effects observed. In general, alcohol is known to have a biphasic effect on social behavior, as low doses increase and high doses decrease the number of social contacts (López-Cruz et al., 2016; López-Cruz, Salamone, & Correa, 2013). Furthermore, mice exposed to alcohol spend more time in interaction with a conspecific than the object, regardless of the dose of alcohol (López-Cruz et al., 2013, 2016).

In contrast, mice exposed to alcohol presented anxiety-like and depression-like signs 24 h after binge drinking, which may correspond for hangover in humans. Hangover is a state that occurs after a single episode of heavy drinking when BAC approaches zero and is associated with a combination of physical signs and affective symptoms, including anxiety and depression. The affective symptoms of hangover – a term used by some authors interchangeably with acute alcohol withdrawal (Marsland et al., 2021; Palmer et al., 2019) – usually emerge at 10 h and may persist even after 24 h following alcohol administration (Karadayian, Busso, Feleder, & Cutrera, 2013; Karadayian & Cutrera, 2013). In accordance, a previous study has already reported that a history of 30 days of binge drinking elicits negative affect in mice, most notably anxiety-like signs, which emerge after 24 h of withdrawal and persist for at least 21 days following the last episode of binge drinking (Lee et al., 2015, 2017). However, in another study previously published, only a weak negative affect, including a few signs of anxiety-like and depression-like behavior, and no elevation of the circulating corticosterone levels, as a biochemical index of stress, were

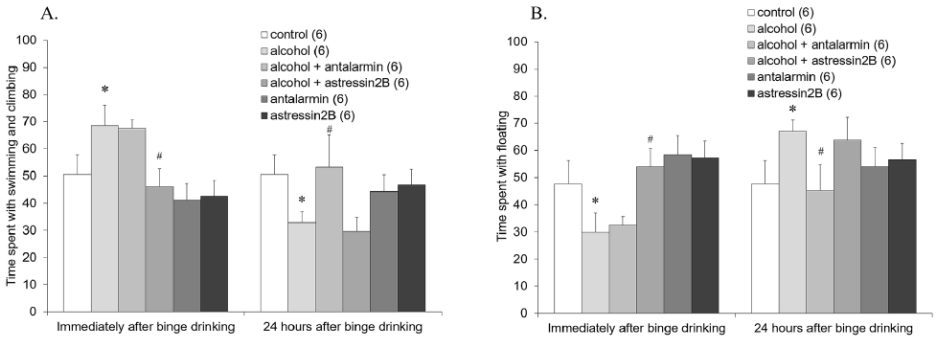

Fig. 2. The effects of binge drinking on the time spent with swimming and climbing (**A**), and floating (**B**) in mice investigated in a forced swim test for signs of depression. Values are presented as means ± SEM; a statistically significant difference was accepted for $p < 0.05$ and indicated with * for alcohol versus control, and # for alcohol + CRF antagonist vs. alcohol alone.

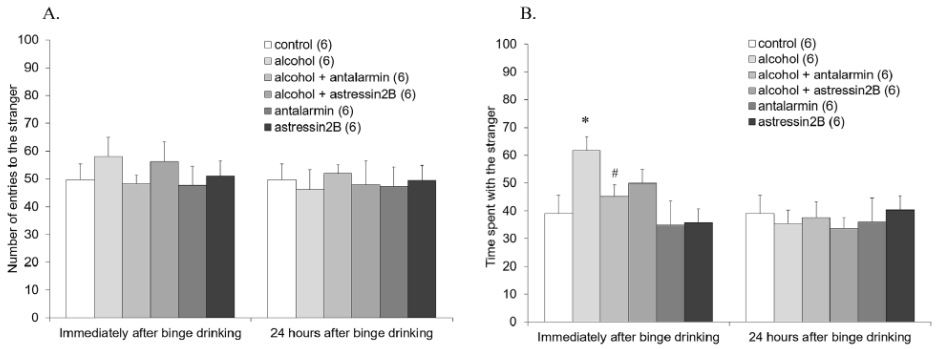

Fig. 3. The effects of binge drinking on the number of entries to (**A**), and the time spent with the stranger (**B**) in mice investigated in a three-chamber social interaction test for their sociability. Values are presented as means ± SEM; a statistically significant difference was accepted for $p < 0.05$ and indicated with * for alcohol vs. control, and # for alcohol + CRF antagonist vs. alcohol alone.

detected after 24 h of binge drinking in mice (Jimenez Chavez et al., 2020). In this study, male and female adolescent and adult mice were subjected to 14 consecutive days of binge drinking using a multi-bottle choice drinking in the dark procedure (Jimenez Chavez et al., 2020). The authors of this study concluded that incubation of negative affect during alcohol withdrawal is age-dependent, and not sex-selective, but also admitted that procedural differences might have accounted for the relatively weak effect of binge drinking on anxiety-like and depressive-like behavior, when compared to other studies (Jimenez Chavez et al., 2020). In addition, binge drinking does not affect the social interaction of male mice, when they are tested 24 h after drinking in the dark. A recent study has already suggested that binge drinking has no impact on the sociability and the preference for social novelty of mice, at least when they are tested 24 h after drinking in the dark (Van Hees et al., 2022). Another study recently published underlined the anxiogenic and cognitive impairing effects of binge drinking (Jimenez Chavez et al., 2022). In this study C57BL/6 mice were exposed to drinking in the dark for a 1-month period and investigated in a battery of behavioral tests, including elevated plus-maze, forced swim, and Morris water-maze tests (Jimenez Chavez et al., 2022). The authors reached the following conclusions: 1) both biological sex and the age of drinking onset are subjective factors that impact voluntary alcohol consumption by mice into old age; 2) binge drinking during later life elicits a negative affective state that is relatively sex-independent; 3) binge drinking during both mature adulthood and old age impairs spatial learning and memory; 4) binge drinking during mature adulthood accelerates deficits in working memory; and 5) mature adult females tend to exhibit more alcohol-induced cognitive impairments than males (Jimenez Chavez et al., 2022). We find these studies very inspiring for our future investigations regarding immediate and persistent effects of binge drinking on male and female mice, at adolescence and adulthood.

The present study also demonstrates that the anxiolytic and antidepressant effects produced by binge drinking are mediated by CRF2, whereas the anxiety-like and depression-like signs observed the next day are promoted by CRF1. This is consistent with the original hypothesis, which proposed that CRF1 and CRF2 play dualistic roles in the brain (Bale, 2014; Bale & Vale, 2004), with CRF1 promoting activation of the hypothalamic-pituitary-adrenal (HPA) axis, anxiety, and depression, and with CRF2 mediating anxiolytic and antidepressant actions. However, a recent hypothesis states that the role of CRF1 and CRF2 in anxiety and depression is not a matter of simple dualism but depends on the brain regions

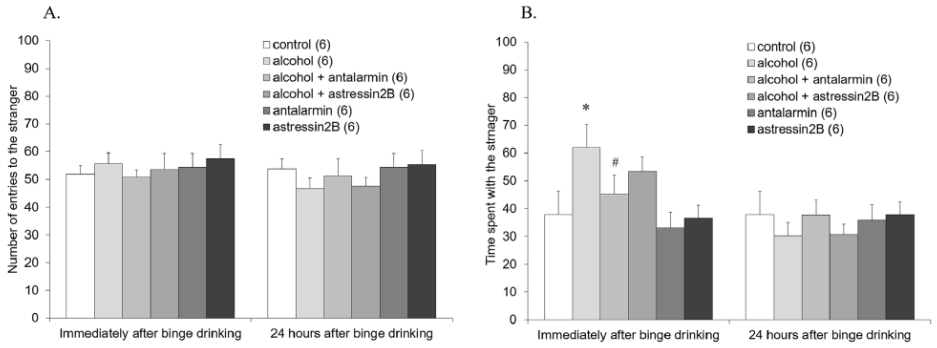

Fig. 4. The effects of binge drinking on the number of entries to (**A**), and the time spent with the stranger (**B**) in mice investigated in a three-chamber social interaction test for their preference for social novelty. Values are presented as means ± SEM; a statistically significant difference was accepted for $p < 0.05$ and indicated with * for alcohol vs. control, and # for alcohol + CRF antagonist vs. alcohol alone.

Table 1
Results of the statistical analysis for elevated plus-maze test.

Number of entries into the open arms		
Groups	Binge drinking	Hangover
Alcohol vs. Control	**$F(5,30) = 9.826$ $p = 0.0063$**	**$F(5,30) = 6.419$ $p = 0.0249$**
Alcohol vs. Alcohol + Antalarmin	$F(5,30) = 9.826$ $p > 0.999$	**$F(5,30) = 6.419$ $p = 0.0006$**
Alcohol vs. Alcohol + Astressin2B	**$F(5,30) = 9.826$ $p = 0.0147$**	$F(5,30) = 6.419$ $p = 0.9311$
Time spent in the open arms		
Groups	**Binge drinking**	**Hangover**
Alcohol vs. Control	**$F(5,30) = 2.263$ $p = 0.0423$**	**$F(5,30) = 2.006$ $p = 0.0440$**
Alcohol vs. Alcohol + Antalarmin	$F(5,30) = 2.263$ $p > 0.999$	**$F(5,30) = 2.006$ $p = 0.0180$**
Alcohol vs. Alcohol + Astressin2B	**$F(5,30) = 2.2630$ $p = 0.0149$**	$F(5,30) = 2.006$ $p = 0.9804$

Table 2
Results of the statistical analysis for forced swim test.

Time spent with swimming and climbing		
Groups	Binge drinking	Hangover
Alcohol vs. Control	**$F(5,30) = 4.295$ $p = 0.0298$**	**$F(5,30) = 1.798$ $p = 0.0508$**
Alcohol vs. Alcohol + Antalarmin	$F(5,30) = 4.295$ P > 0.999	**$F(5,30) = 1.798$ $p = 0.0365$**
Alcohol vs. Alcohol + Astressin2B	**$F(5,30) = 4.295$ $p = 0.0107$**	$F(5,30) = 1.798$ $p = 0.994$
Time spent with floating		
Groups	**Binge drinking**	**Hangover**
Alcohol vs. Control	**$F(5,30) = 4.223$ $p = 0.0335$**	**$F(5,30) = 1.429$ $p = 0.0486$**
Alcohol vs. Alcohol + Antalarmin	$F(5,30) = 4.223$ $p = 0.9996$	**$F(5,30) = 1.429$ $p = 0.3547$**
Alcohol vs. Alcohol + Astressin2B	**$F(5,30) = 4.223$ $p = 0.0870$**	$F(5,30) = 1.429$ $p = 0.9996$

and neuron populations being activated (Henckens, Deussing, & Chen, 2016; Janssen & Kozicz, 2013). Therefore, future experiments using modern techniques of CRF overexpression and global or local CRF1 and CRF2 knockout animal models should determine the intimate brain regions and mechanisms involved in binge drinking. Our pre-clinical study may have clinical implications. A previous study demonstrated that pre-treatment with a CRF1 antagonist or CRF2 agonist prior to alcohol self-administration could reduce the amount of alcohol administered (Lowery et al., 2010). The present study using the same animal model suggests that pre-treatment with a selective CRF1 antagonist and a selective CRF2 antagonist could attenuate both the positive, rewarding effects, and the negative, aversive effects of alcohol and alcohol withdrawal, respectively. In this order of thought, co-administration of these drugs might prevent spiraling of repeated cycles of binge drinking into alcohol addiction. In addition, selective CRF2 agonists, such as urocortin 2 and urocortin 3, may also prove useful in the therapy of alcohol addiction, since our previous study revealed that these neuropeptides ameliorate the anxiety- and depression-like state developed during nicotine addiction, as well (Bagosi et al., 2016).

Many other investigators have examined the role of CRF1 and CRF2 in binge or heavy drinking of alcohol (Albrechet-Souza et al., 2015; Kaczmarek, 2017; Kaur, Li, Stenzel-Poore, & Ryabinin, 2012; Sparta et al., 2013). We believe that any inconsistencies found between our study and others investigating the anxiety-like, depression-like, and social behavior using the same animal model of binge drinking could be due to the changes in the drinking in the dark paradigm. In our experiments, C57BL/6 mice were exposed to alcohol for 4 days, according to the classical drinking in the dark paradigm, even if the animals did not always reach the BAC of 0.08 g/dL within 2 h (Thiele et al., 2014; Thiele & Navarro, 2014). In comparison, in other experiments the mice were exposed repeatedly to alcohol, at different times, and for longer periods in order to reach the alcohol level that is characteristic for binge drinking (Lee et al., 2015, 2017; Van Hees et al., 2022). As regards the robust negative affect that was observed 24 h after a single session of binge drinking in our case, and that was described after several cycles of binge drinking and withdrawal in other cases, we presume that these may also arise from the different methodology. In our experiments, mice with BACs lower than 0.08 g/dL were excluded from the statistical analysis

Table 3
Results of the statistical analysis for social interaction test (sociability).

Number of entries to the stranger		
Groups	Binge drinking	Hangover
Alcohol vs. Control	$F(5,30) = 0.5843$ $p = 0.8988$	$F(5,30) = 0.1044$ $p = 0.9987$
Alcohol vs. Alcohol + Antalarmin	$F(5,30) = 0.5843$ $p = 0.8175$	$F(5,30) = 0.1044$ $p = 0.9863$
Alcohol vs. Alcohol + Astressin2B	$F(5,30) = 0.5843$ $p > 0.999$	$F(5,30) = 0.1044$ $p > 0.999$
Time spent with the stranger		
Groups	**Binge drinking**	**Hangover**
Alcohol vs. Control	**$F(5,30) = 3.782$ $p = 0.049$**	$F(5,30) = 0.1693$ $p > 0.999$
Alcohol vs. Alcohol + Antalarmin	**$F(5,30) = 3.782$ $p = 0.0261$**	$F(5,30) = 0.1693$ $p = 0.998$
Alcohol vs. Alcohol + Astressin2B	$F(5,30) = 3.782$ $p = 0.6125$	$F(5,30) = 0.1693$ $p > 0.999$

corticotropin-releasing factor receptors 1 and 2 in the ventral tegmental area. *Biological Psychiatry, 81*(11), 905–906.
Kaidanovich-Beilin, O., Lipina, T., Vukobradovic, I., Roder, J., & Woodgett, J. R. (2011). Assessment of social interaction behaviors. *Journal of Visualized Experiments*, (48), 2473.
Karadayian, A. G., Busso, M. J., Feleder, C., & Cutrera, R. A. (2013). Alterations in affective behavior during the time course of alcohol hangover. *Behavioural Brain Research, 253*, 128–138.
Karadayian, A. G., & Cutrera, R. A. (2013). Alcohol hangover: Type and time-extension of motor function impairments. *Behavioural Brain Research, 247*, 165–173.
Kaur, S., Li, J., Stenzel-Poore, M. P., & Ryabinin, A. E. (2012). Corticotropin-releasing factor acting on corticotropin-releasing factor receptor type 1 is critical for binge alcohol drinking in mice. *Alcoholism: Clinical and Experimental Research, 36*(2), 369–376.
Kent, K., Butler, K., & Wood, R. I. (2014). Ethanol induces conditioned social preference in male mice. *Alcoholism: Clinical and Experimental Research, 38*(4), 1184–1192.
Koob, G. F. (2013). Theoretical frameworks and mechanistic aspects of alcohol addiction: Alcohol addiction as a reward deficit disorder. *Current Topics in Behavioral Neurosciences, 13*, 3–30.
Koob, G. F. (2014). Neurocircuitry of alcohol addiction: Synthesis from animal models. *Handbook of Clinical Neurology, 125*, 33–54.
Lee, K. M., Coehlo, M., McGregor, H. A., Waltermire, R. S., & Szumlinski, K. K. (2015). Binge alcohol drinking elicits persistent negative affect in mice. *Behavioural Brain Research, 291*, 385–398.
Lee, K. M., Coehlo, M. A., Solton, N. R., & Szumlinski, K. K. (2017). Negative affect and excessive alcohol intake incubate during protracted withdrawal from binge-drinking in adolescent, but not adult, mice. *Frontiers in Psychology, 8*, 1128.
Lister, R. G. (1987). The use of a plus-maze to measure anxiety in the mouse. *Psychopharmacology (Berl), 92*(2), 180–185.
López-Cruz, L., Salamone, J. D., & Correa, M. (2013). The impact of caffeine on the behavioral effects of ethanol related to abuse and addiction: A review of animal studies. *Journal of Caffeine Research, 3*(1), 9–21.
López-Cruz, L., San-Miguel, N., Bayarri, P., Baqi, Y., Müller, C. E., Salamone, J. D., et al. (2016). Ethanol and caffeine effects on social interaction and recognition in mice: Involvement of adenosine A(2A) and A(1) receptors. *Frontiers in Behavioral Neuroscience, 10*, 206.
Lowery, E. G., Spanos, M., Navarro, M., Lyons, A. M., Hodge, C. W., & Thiele, T. E. (2010). CRF-1 antagonist and CRF-2 agonist decrease binge-like ethanol drinking in C57BL/6J mice independent of the HPA axis. *Neuropsychopharmacology, 35*(6), 1241–1252.
Marsland, P., Parrella, A., Vore, A. S., Barney, T. M., Varlinskaya, E. I., & Deak, T. (2021). Male, but not female, Sprague Dawley rats display enhanced fear learning following acute ethanol withdrawal (hangover). *Pharmacology, Biochemistry and Behavior, 208*, Article 173229.
Olney, J. J., Marshall, S. A., & Thiele, T. E. (2018). Assessment of depression-like behavior and anhedonia after repeated cycles of binge-like ethanol drinking in male C57BL/6J mice. *Pharmacology, Biochemistry and Behavior, 168*, 1–7.
Palmer, E., Tyacke, R., Sastre, M., Lingford-Hughes, A., Nutt, D., & Ward, R. J. (2019). Alcohol hangover: Underlying biochemical, inflammatory and neurochemical mechanisms. *Alcohol and Alcoholism, 54*(3), 196–203.
Paxinos, G., & Franklin, K. B. J. (2004). *The mouse brain in stereotaxic coordinates* (2nd ed.). Amsterdam; Boston: Elsevier Academic Press.
Porsolt, R. D., Bertin, A., & Jalfre, M. (1977). Behavioral despair in mice: A primary screening test for antidepressants. *Archives Internationales de Pharmacodynamie et de Therapie, 229*(2), 327–336.
Reul, J. M., & Holsboer, F. (2002). On the role of corticotropin-releasing hormone receptors in anxiety and depression. *Dialogues in Clinical Neuroscience, 4*(1), 31–46.
Rhodes, J. S., Best, K., Belknap, J. K., Finn, D. A., & Crabbe, J. C. (2005). Evaluation of a simple model of ethanol drinking to intoxication in C57BL/6J mice. *Physiology & Behavior, 84*(1), 53–63.
van Schrojenstein Lantman, M., van de Loo, A. J., Mackus, M., & Verster, J. C. (2016). Development of a definition for the alcohol hangover: Consumer descriptions and expert consensus. *Current Drug Abuse Reviews, 9*(2), 148–154.
Sparta, D. R., Hopf, F. W., Gibb, S. L., Cho, S. L., Stuber, G. D., Messing, R. O., et al. (2013). Binge ethanol-drinking potentiates corticotropin releasing factor R1 receptor activity in the ventral tegmental area. *Alcoholism: Clinical and Experimental Research, 37*(10), 1680–1687.
Thiele, T. E., Crabbe, J. C., & Boehm, S. L., 2nd (2014). "Drinking in the dark" (DID): A simple mouse model of binge-like alcohol intake. *Current Protocols in Neuroscience, 68*, 9.49.1–9.49.12.
Thiele, T. E., & Navarro, M. (2014). "Drinking in the dark" (DID) procedures: A model of binge-like ethanol drinking in non-dependent mice. *Alcohol, 48*(3), Article 235241.
Van Hees, L., Didone, V., Charlet-Briart, M., Van Ingelgom, T., Alexandre, A., Quertemont, E., et al. (2022). Voluntary alcohol binge-drinking in adolescent C57Bl6 mice induces delayed appearance of behavioural defects in both males and females. *Addiction Biology, 27*(1), Article e13102.
Wood, R. I., Knoll, A. T., & Levitt, P. (2015). Social housing conditions and oxytocin and vasopressin receptors contribute to ethanol conditioned social preference in female mice. *Physiology & Behavior, 151*, 469–477.