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

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PUBLICATIONS

1. Original publications the PhD thesis 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 PhD thesis:

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 PhD thesis:

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 PhD thesis:

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)

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ABBREVIATIONS

[^{3}H] = tritium
ACTH = adrenocorticotropic hormone
AVP = arginine vasopressin
BAC = blood alcohol concentration
BNST = bed nucleus of stria terminalis
CEA = central nucleus of the amygdala
CNS = central nervous system
CORT = corticosterone
CRF = corticotropin-releasing factor
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
ELISA = enzyme-linked immunosorbent assay
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
IV = intravenous
LC = locus coeruleus
NACC = nucleus accumbens
PCR = polymerase chain reaction
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. The major role CRF is to mediate the neuroendocrine, autonomic and behavioral responses to stress. 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. 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. 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. 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.

The effects of CRF are mediated through two different CRF receptors, CRF1 and CRF2. 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: CRF1 has α and β isoforms in addition to subtypes designated c-h, which have been detected in rodent and human tissues, whereas CRF2 has three functional subtypes, α, β, and γ. CRF2α and CRF2β have been detected in rodents, primates and humans, while CRF2γ has only been reported in humans.

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. 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.

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

Urocortin 1 (UCN1) is a 40 amino acid neuropeptide that was isolated first from rat brain in 1995. The name urocortin derives from the fish urotensin (63% homology) and the mammalian corticotropin(-releasing factor) (45% homology). 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.

Urocortin 2 (UCN2) is a 38 amino acid neuropeptide that was identified first in mouse brain in 2001. In humans, it is also known as stresscopin-related peptide (SRP) (34% homology with CRF). 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.

Urocortin 3 (UCN3) is another 38 amino acid neuropeptide identified in the mouse brain in 2001. In humans, it is also known as stresscopin (SCP) (36% homology with CRF). 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.

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. In the CNS, CRF1 is distributed abundantly the cerebral cortex, cerebellum, and anterior pituitary, whereas CRF2 is limited to subcortical regions, such as the hypothalamus, hippocampus, amygdala, and posterior pituitary.

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

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, whereas activation of CRF2 by administration of UCN2 and UCN3 produced anxiolytic and antidepressant effects in rats. 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. Therefore, the exact role of CRF1 and CRF2 in the activation of the HPA axis, anxiety and depression is still under debate.

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. 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. 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. The first truly selective antagonists were CP-154,526 and its structural analog antalarmin. These are non-peptidic, competitive and selective CRF1 antagonists, which were could attenuate stress, anxiety-like and depression-like behavior in rodents. 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. Antisauvagine and astressin2B are peptidic, competitive and selective CRF2 antagonists, which are usually administered peripherally. 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).

Unfortunately, these drugs that seemed promising in animal experiments, were ineffective in humans. 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.

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

Binge drinking itself is defined as consuming a large amount of alcohol in a short period of time. A large amount of alcohol refers to five or more standard alcoholic drinks in men and four or more standard alcoholic drinks in women 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. Hangover is a temporary state described as the unpleasant next-day effects after binge drinking. 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. Alcohol addiction is best resembled by alternating episodes of binge drinking and hangover. 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. Repeating episodes of binge drinking may elicit persistent negative affect, including anxiety and depression, and alteration of social behavior similar to that described during acute alcohol withdrawal. 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.

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. 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. 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.

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. 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. During alcohol intoxication the reward system is activated. 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. Alcohol intoxication may also result in amnesia that can be related to the reduction of the hippocampal glutamate (GLU) release. 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. 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. The alcohol withdrawal syndrome consists of somatic (physical) signs and affective (emotional) symptoms that emerge immediately after alcohol cessation. 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. 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.

2. AIM OF STUDY

The first aim of the presnt 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. 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.

The rats were also implanted with a stainless steel Luer cannula, aimed at the right lateral cerebral ventricle under anesthesia with 35 mg/kg pentobarbital sodium. 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.

3.3. Substances

For drinking in the dark procedure tap water alcohol solution of 20%, for the intraperitoneal (IP) treatment saline and alcohol solution of 20%, for the intracerebroventricular (ICV) pretreatment saline solution, antalarmin and astressin2B were used. For the behavioral tests tap water and sodium hypochlorite solution, for the laboratory tests commercially available enzymatic kits (BAC kit, CRF, AVP and ACTH sandwich ELISA kits, GeneJET RNA Purification Kit and Maxima First Strand cDNA Synthesis Kit) were used. For the determination of plasma CORT concentration methylene chloride, sulfuric acid and alcohol solution were used. For *in vitro* homogenization of the hypothalamus acetic acid, for *in vitro* superfusion of the striatum, amygdala and hippocampus Krebs solution and tritium-labelled neurotransmitters, including [3H]DA, [3H]GABA and [3H]GLU, and Ultima Gold scintillation fluid were used.

3.4. Treatments

The mice were exposed to drinking in the dark, a classical animal model for binge drinking. 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. 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. 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. 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. 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.

3.5.2. Forced swim test

The mice were also investigated in a forced swim test described first by Porsolt. 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.

3.5.3. Social interaction test

The mice were also tested in a social interaction arena invented by Crawley. 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. In both tests the following parameters were recorded by an observer sitting at 200 cm distance from the box for a 5 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.

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 blood alcohol concentration (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. The intake of this amount of alcohol should produce a BAC of 0.08 g/dL within 2 hours in C57BL/6 mice. 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. 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. 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. 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, 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 and then homogenized. On the following day, GeneJET RNA Purification Kit was used according to the manufacturer's instructions. The first strand cDNA was synthesized using the Maxima First Strand cDNA Synthesis Kit according to the manufacturer's instructions.

3.6.3. Enzyme-Linked Immunosorbent Assay (ELISA)

For the determination of the hypothalamic CRF and AVP and plasma ACTH concentration an *in vitro* homogenization was performed that was followed by sandwich ELISA according to the manufacturer's instructions. The hypothalamus was isolated from each rat according to the stereotaxic atlas of the rat brain, as previously described, and then homogenized. The concentration of hypothalamic CRF and AVP and plasma ACTH 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. 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. 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, 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; RC 0.0 to - 2.0 mm, ML + 3.0 to + 6.0 mm, DV +7.0 to + 10.0 mm for the amygdala; 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. The brain tissue was cut into slices and the brain slices obtained were incubated with tritium-labelled neurotransmitters. The radioactivity was measured with a liquid scintillation spectrometer and expressed in count per minute (CPM).

3.7. Statistical analysis

Statistical analysis of the results was performed by analysis of variance (GraphPad Prism, GraphPad Software Inc., USA) in case of mice. The differences between groups were determined by one-way ANOVA, followed by Tukey's post-hoc test for pair-wise comparisons. Statistical analysis of the results was performed by ANOVA (SPSS Inc., USA) in case of rats. A two-way 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. 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.

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. 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.

Hypothalamic CRF mRNA expression of rats was increased by alcohol intoxication and withdrawal, and these stimulatory effects were reduced by antalarmin, but not astressin2B. 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. Hypothalamic AVP mRNA expression of rats was decreased by alcohol intoxication and withdrawal, but these inhibitory effects were not influenced significantly either by antalarmin or astressin2B. 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. Plasma ACTH level of rats was elevated by alcohol intoxication and withdrawal, and these elevations were ameliorated by antalarmin, but not astressin2B.

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. Alcohol intoxication increased, whereas alcohol withdrawal decreased the striatal DA release, and both effects were reduced by antalarmin, but not astressin2B. 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. 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.

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. 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. 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. Furthermore, mice exposed to alcohol spend more time in interaction with a conspecific than the object, regardless of the dose of alcohol.

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 - usually emerge at 10 hours and may persist even after 24 hours following alcohol administration. 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. 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. 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. 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. 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. Another study recently published underlined the anxiogenic and cognitive impairing effects of binge drinking. 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. 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.

Lately, there are several studies investigating the negative affect induced by binge or heavy drinking of alcohol. 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. 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.

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. 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. 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. The amygdalar GABA is presumed to play role in the positive, anxiolytic effects of alcohol. 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. 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. 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.

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, 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.

A previous study reported that pretreatment with CRF1 antagonist or CRF2 agonist prior to alcohol self-administration could reduce the amount of alcohol administered. 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.

Previous studies provided a solid base of evidence to support the role of CRF in the alcohol-induced activation of the HPA axis. First, administration of a CRF antiserum or a CRF antagonist inhibited the stimulatory effect of alcohol on ACTH secretion in rats. Second, bilateral destruction of the paraventricular CRF-secreting neurons ameliorated, although did not abolish, the alcohol-stimulated ACTH secretion. 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. 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. First, administration of an AVP antiserum or an AVP antagonist inhibited the stimulatory effect of alcohol on ACTH secretion in rats. Second, removal of endogenous AVP in rats previously exposed to bilateral destruction of the paraventricular neurons diminished partially the alcohol-stimulated ACTH secretion. Third, administration of alcohol increased the expression of AVP heteronuclear RNA and mRNA levels. 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, and the alcohol-induced ACTH secretion could be blocked by non-selective CRF receptor antagonist astressin and selective CRF1 antagonist NBI 30775. In contrast, alcohol administration was unable to produce up-regulation of CRF2 expression in the PVN, and the alcohol-induced ACTH secretion could not be blocked by selective CRF2 antagonist astressin2B.

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. 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. 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.

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. 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.

6. CONCLUSIONS

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

Our experiments in mice demonstrate 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 experiments in rats demonstrate 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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