THE ROLE OF THE SELECTIVE CRF RECEPTOR AGONISTS AND ANTAGONISTS IN NICOTINE ADDICTION

Candidate: András Buzás, M.D.
Department of Pathophysiology, University of Szeged
Department of Surgery, University of Szeged

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Supervisor: Zsolt Bagosi, M.D., Ph.D.
Doctoral School of Theoretical Medicine, University of Szeged

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CONTENTS

PUBLICATIONS

1. Original publications the present work is based on:

I. **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; 1706: 41–47.) **IF: 3.125**

II. 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; 1652:21-29.) **IF: 2.746**

2. Conference presentations related to the present work:

I. **Buzás A**, Bokor P, Bagosi Z, Palotai M, Jászberényi M, Csabafi K, Szabó G. The effects of selective CRF receptor antagonists in alcohol-treated rats (HMAA, 2013, Balatonfüred, Hungary)

II. Bokor P, **Buzás A**, Bagosi Z, Palotai M, Jászberényi M, Csabafi K, Szabó G. The effects of urocortin II and urocortin III on the anxiety- and depression-like symptoms in nicotine-treated mice (HMAA, 2013, Balatonfüred, Hungary)

3. Poster presentations related to the present work:

I. Bagosi Z, Palotai M, **Buzás A**, Bokor P, Csabafi K, Szabó G. The effects of selective CRF receptor antagonists in rats following chronic alcohol treatment and acute alcohol withdrawal (MITT, Budapest, Hungary, 2013)

II. Bagosi Z, Bokor P, **Buzás A**, Palotai M, Jászberényi M, Csabafi K, Szabó G. The effects of urocortin II and urocortin III on the anxiety- and depression-like symptoms in nicotine-treated mice (MÉT, Budapest, Hungary, 2013)

III. Bagosi Z, Palotai M, **Buzás A**, Bokor P, Jenei A, Csabafi K, Jászberényi M, Telegdy G, Szabó G. Role of the hypothalamic CRF and AVP in mediating the activation of the HPA axis in alcohol-treated and alcohol-deprived rats (FEPS, Budapest, Hungary, 2014)

IV. 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)

V. Bagosi Z, Bokor P, **Buzás A**, Balangó B, Pintér D, Csabafi K, Szabó G. The effects of the selective CRF2 receptor agonists in mice exposed to chronic nicotine treatment and consequent acute withdrawal (FAMÉ, Pécs, Hungary, 2016)

VI. Bagosi Z, Balangó B, Pintér D, Bokor P, **Buzás A**, Csabafi K, Szabó G. The effects of selective CRF receptor antagonists in rats exposed to chronic nicotine treatment and consequent acute withdrawal (FENS, Pécs, Hungary, 2017)

VII. Bagosi Z, Karasz G, **Buzás A**, Csabafi K, Telegdy G, Szabó G. The effects of selective CRF receptor antagonists on the affective signs of binge drinking (MÉT, Szeged, Hungary, 2018)

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TOTAL CITATION: 5 (WITHOUT SELF-CITATION: 4)

ABBREVIATIONS

[^{3}H] = tritium
ACTH = adrenocorticotropic hormone
AVP = arginine vasopressin
BNST = bed nucleus of the stria terminalis
CeA = central nucleus of the amygdala
CNS = central nervous system
CRF = corticotropin-releasing factor
CRF-BP = corticotropin-releasing factor-binding protein
CRFR1 = corticotropin-releasing factor receptor type 1
CRFR2 = corticotropin-releasing factor receptor type 2
CRFRs = corticotropin-releasing factor receptors
CRH = corticotropin-releasing hormone
DSM-5 = Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition
EWN = Edinger-Westphal nucleus
GABA = gamma-aminobutyric acid
GI = gastrointestinal
GPCR = G protein-coupled receptor
HPA = hypothalamic-pituitary-adrenal
IBD = inflammatory bowel diseases
IBS = irritable bowel syndrome
ICV = intracerebroventricular
IP = intraperitoneal
LC = locus coeruleus
NA = noradrenaline
NAcc = nucleus accumbens
nAChR = nicotinic acetylcholine receptor
PTSD = post-traumatic stress disorder
PVN = paraventricular nucleus
SC = subcutaneous
SCP = stresscopin
SNS = sympathetic nervous system
SRP = stresscopin-related peptide
SVG = sauvagine
UCN = urocortin
URO = urotensin
VTA = ventral tegmental area
WHO = World Health Organization

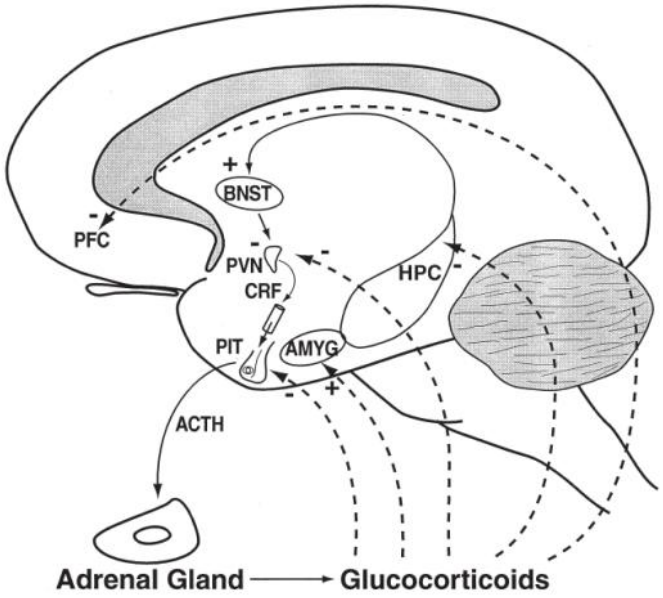
1. INTRODUCTION

1.1. CRF and CRF receptors

Corticotropin-releasing hormone (CRH) or corticotropin-releasing factor (CRF) is a 41 amino acid neuropeptide. It is also known as corticoliberin, because it is the major neurohormone that stimulates the release of adrenocorticotropic hormone (ACTH) or corticotropin, along with the synergistic arginin-vasopressin (AVP), both being released from the paraventricular nucleus (PVN) of the hypothalamus [1-2]. CRF is also an important extrahypothalamic neurotransmitter that is released from the medulla oblongata and the central nucleus of the amygdala (CeA) [3-4]. Thus, CRF is expressed mainly in the central nervous system (CNS), but it is also found in the periphery: in the gastrointestinal (GI) tract, skin, and adrenal gland [3-4]. The main role of CRF is to mediate the neuroendocrine, autonomic and behavioral stress responses [3-4]. The neuroendocrine stress response is represented by the activation of the hypothalamic-pituitary adrenal (HPA) axis that is mediated by CRF released from the PVN in order to stimulate the release of ACTH from the anterior pituitary and the subsequent release of glucocorticoids (corticosterone in rodents, cortisol in humans) from the adrenal cortex [3-4]. The autonomic stress response is represented by the activation of the sympathetic nervous system (SNS) that is mediated by CRF released from the medulla oblongata which, in turn, stimulates the locus coeruleus (LC) noradrenaline (NA) system, and eventually the release of adrenaline from the adrenal medulla [3-4]. 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 activity which are mediated among others by amygdalar CRF [3-4] **(Figure 1)**.

The actions of CRF are mediated *via* two different CRF receptors (CRFRs), CRFR1 and CRFR2, which are two proteins of 415 and 397-437 amino acids, respectively [5-6]. CRFRs belong to the class B subtype of G protein–coupled receptor (GPCR) superfamily and they are produced from distinct genes and have several splice variants expressed on the surface of various central and peripheral tissues [7]. CRFR1 has α and β isoforms in addition to subtypes designated c-h, which have been detected in rodent and human tissues [8]. CRFR2 is expressed in three functional subtypes, α, β, and γ [8]. CRFR2α and CRFR2β have been detected in rodents, primates and humans, but CRFR2γ has only been reported in humans [8].

A.



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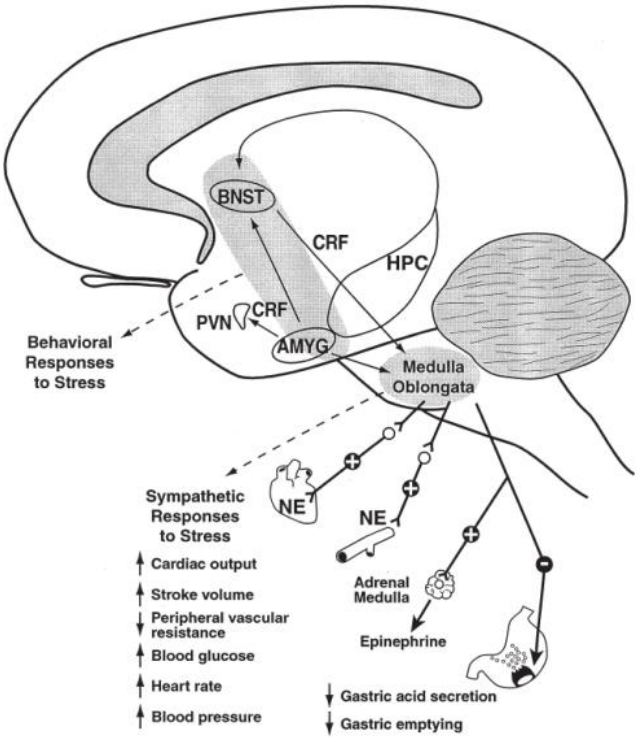


Figure 1. The role of the hypothalamic **(A)** and extrahypothalamic CRF **(B)** [9]

Both CRFRs are found in the CNS and the periphery, although CRFR1 is expressed more abundantly in the CNS, whereas CRFR2 is more dominant in the periphery [10]. In the CNS CRFR1 is distributed throughout the cerebral cortex, cerebellum, olfactory bulb, medial septum, hippocampus, amygdala, and anterior pituitary [11]. CRFR2 is limited centrally to subcortical regions, such as the lateral septum, hippocampus, amygdala, hypothalamus, and posterior pituitary [11]. In addition, both CRFRs are expressed in the ventral and dorsal striatum [11]. Initially, it was presumed that CRFR1 and CRFR2 promote antagonistic effects in the CNS, since activation of CRFR1 induced activation of the HPA axis, anxiety, depression, and locomotor hyperactivity (at least in a familial environment), whereas activation of CRFR2 produced anxiolytic, antidepressant, and locomotor suppressive effects [1, 3-4] **(Figure 2)**. Recently, it was proposed that the role of CRFR1 and CRFR2 in the stress response is not a matter of simple dualism, but it depends upon the brain regions and neuron populations being activated [12-13] **(Figure 3)**.

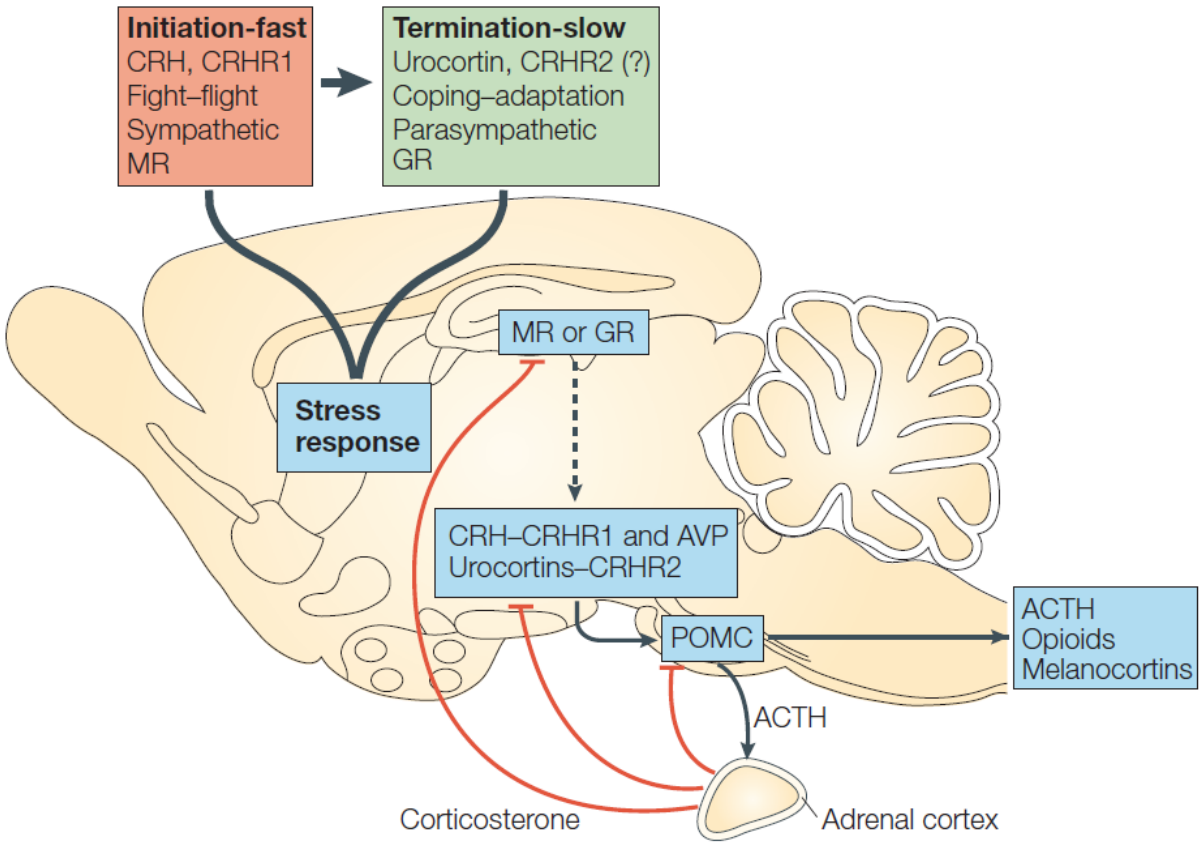


Figure 2. The role of CRFR1 and CRFR2 in the stress response (the classic theory) [14]

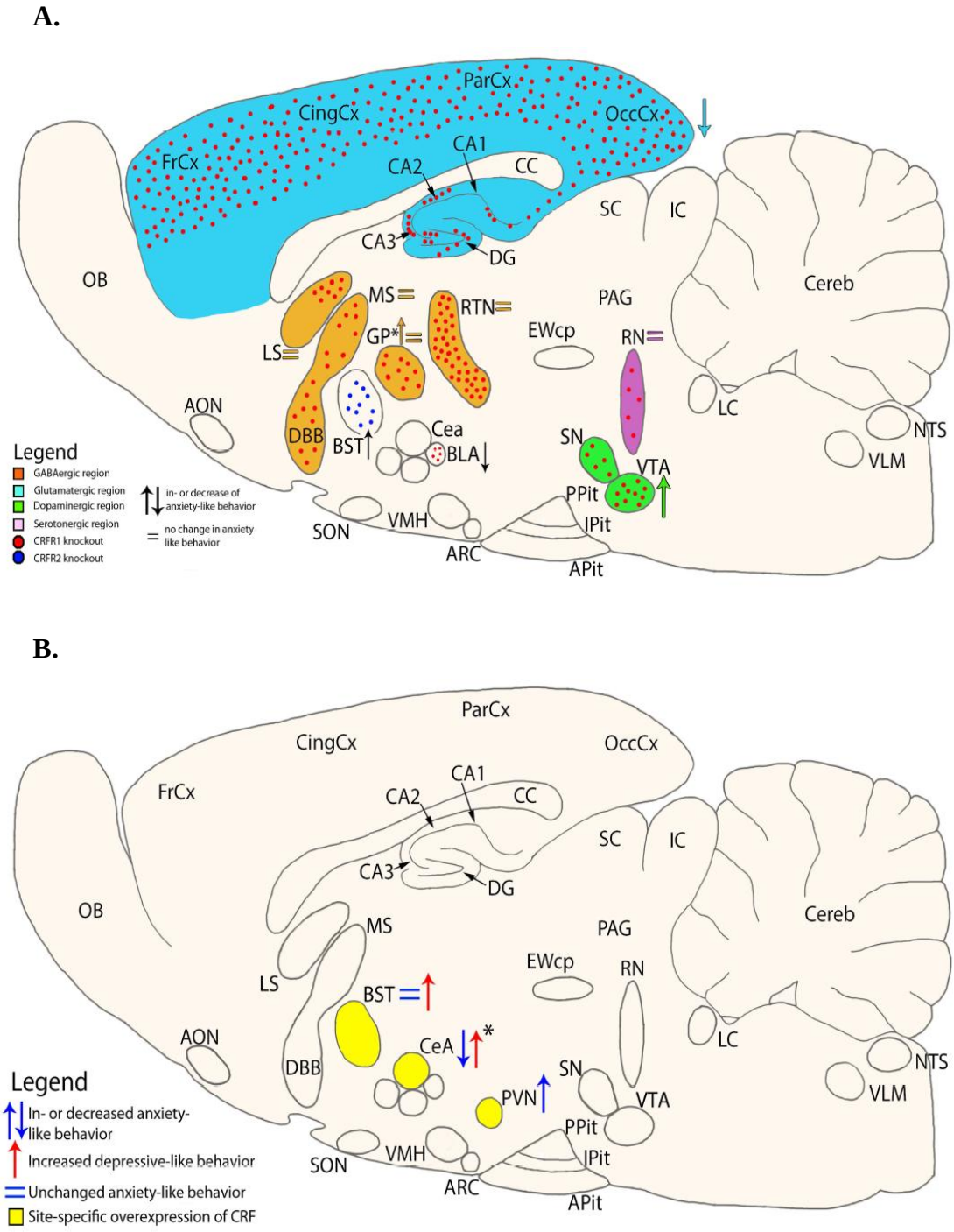


Figure 3. The role of CRFR1 and CRFR2 in anxiety and depression (the new theory) [13]

1.2. CRF receptor agonists

Since CRF has been isolated [1], a new family of CRF-like peptides, termed urocortins were discovered. The name urocortin derives from the frog analogue of CRF urotensin (URO) and CRF or corticoliberin itself [15]. Thus, today the mammalian family of CRF-related peptides consists of three ligands: urocortin I (UCN I) [16], urocortin II (UCN II) [17], and urocortin III (UCN III), two receptors: CRFR1 and CRFR2, and one binding protein: CRF-BP [15]. In humans, UCN II is also known as stresscopin-related peptide (SRP), while UCN III is known as stresscopin (SCP) [18].

The urocortins have similar chemical structures, but different anatomical distribution, pharmacological properties and physiological functions, when comapred to CRF [19-20] **(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.

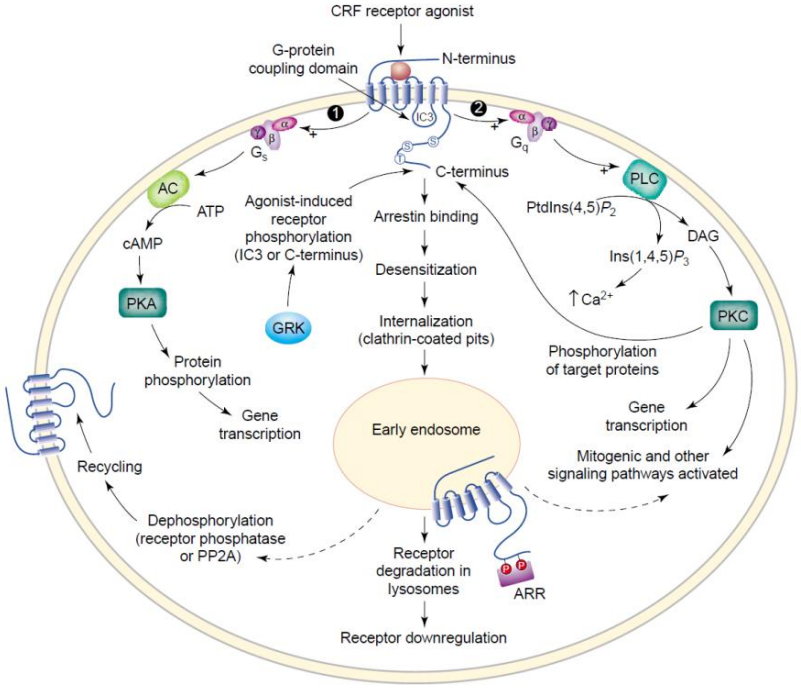
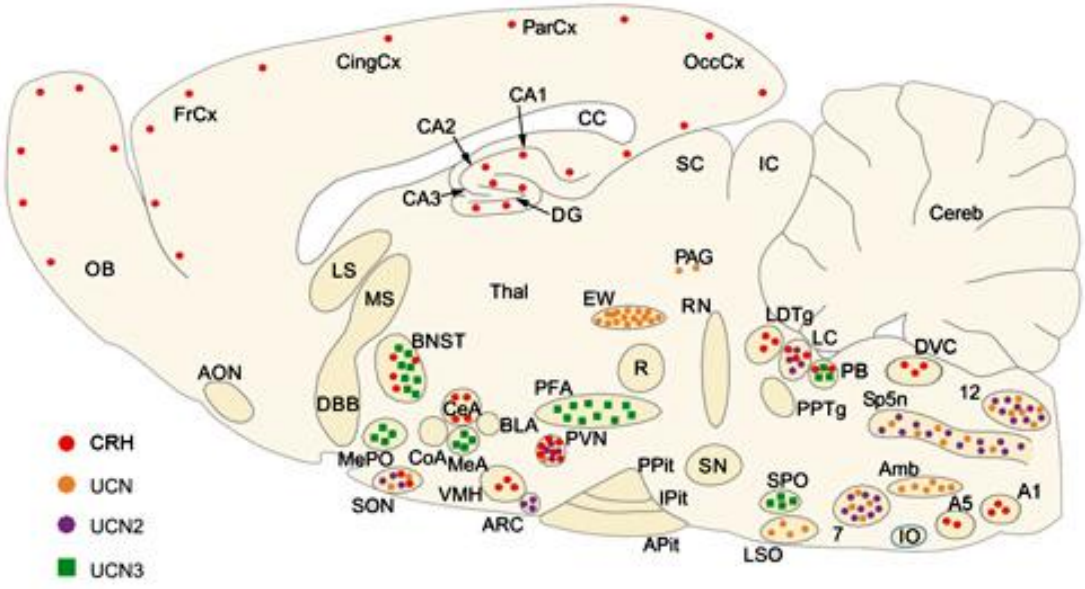


Figure 4. The chemical structure **(A)** and intracellular signalization **(B)** of CRFR agonists [15]

As aforementioned, CRF is a 41 aminoacid neuropeptide isolated first from ovine brain that is expressed predominently in the PVN and the CeA, but it was also found in the periphery: the GI tract, skin, and adrenal gland [21-23]. CRF fibers were shown in the internal layer of the median eminence, lateral septum, central amygdala, bed nucleus of the stria terminalis (BNST), raphe nuclei and spinal cord [21-23]. In contrast, UCN I is a 40 amino acid neuropeptide that was identified for the first time in rat midbrain [16]. It is expressed prominently in the Edinger–Westphal nucleus (EWN), rostroventral midbrain, supraoptic nucleus of the hypothalamus and superior lateral olive [24-28]. Dense UCN I fibers were found in the the internal layer of the median eminence, lateral septum, dorsal raphe nucleus and spinal cord, with scattered fibers in the hypothalamus, hippocampus, cerebral cortex, and posterior pituitary [21, 23-24, 27-28]. In the periphery UCN I was shown in the GI tract, testis, cardiac myocytes, thymus, skin, and spleen [19-20]. UCN II is a 38 amino acid neuropeptide that was cloned from the mouse brain and named SRP in humans [17]. UCN II is expressed in the PVN, arcuate nucleus of the hypothalamus and LC [17, 27, 29]. The terminals of the UCN II fibers are unknown, since no reliable UCN II immunohistochemistry has been performed yet. In the periphery UCN II was found in the heart, blood cells, and adrenal gland [19-20]. UCN III is another 38 amino acid neuropeptide that was cloned from the mouse brain and named SCP in humans [18]. UCN III is expressed in the forebrain regions, the preoptic nucleus and perifornical region of the hypothalamus and the medial nucleus of the amygdala [18, 30]. UCN III fiber terminals were found in the ventromedial hypothalamus, lateral septum, BNST and the medial nucleus of the amygdala [18, 30]. In the periphery UCN III was found in the GI tract and pancreas [19-20] **(Figure 5)**.

CRF has tenfold higher affinity for CRFR1 than for CRFR2, while UCN I show equal affinity for both CRFRs. In contrast, UCN II and UCN III have 1000 fold higher affinity for CRFR2, therefore they are considered selective agonists of CRFR2 [15]. In addition, CRF and UCN I can be found attached to CRF-BP [20, 31]. CRF-BP is a 37-kDa N-linked glycoprotein expressed in the brain and pituitary of rodents, primates and humans [7, 32]. In rats CRF-BP has been demonstrated to block CRF-induced ACTH secretion from rat anterior pituitary cells [7, 32]. In humans, CRF-BP was detected in the liver and in the circulation [7, 32]. It was suggested that CRF-BP may inhibit the activation of the HPA axis under different conditions, such as pregnancy [7, 32]. CRF-BP is also located in brain regions which are not associated with CRF activity, locations which suggest that it may also have CRF-independent functions, such as feeding and cognition [7, 32] **(Figure 6)**.

A.



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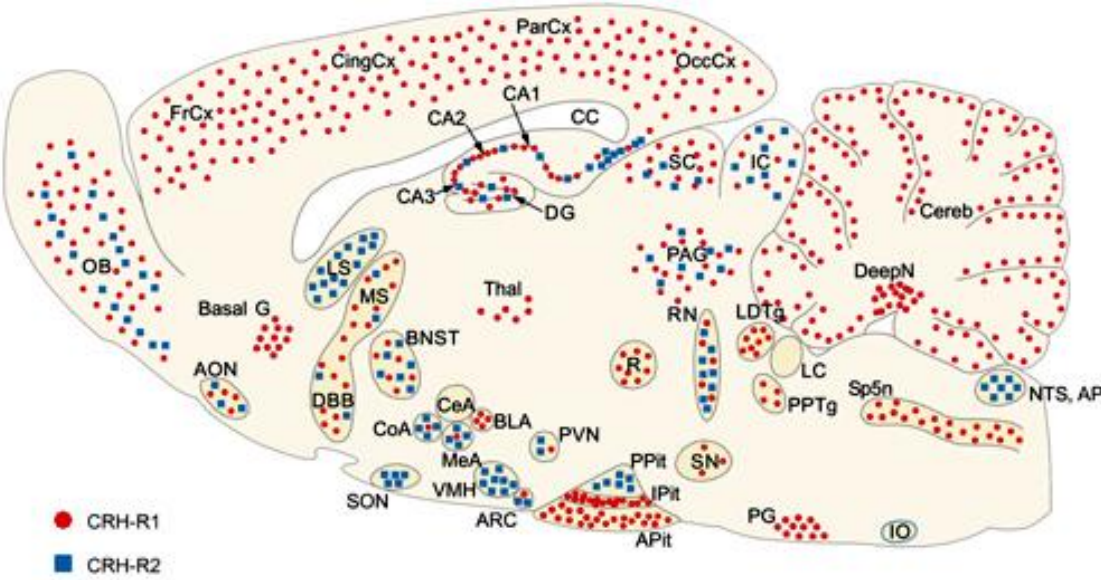

Figure 5. The anatomical distribution of the CRFR agonists **(A)** and CRFRs **(B)** [33]

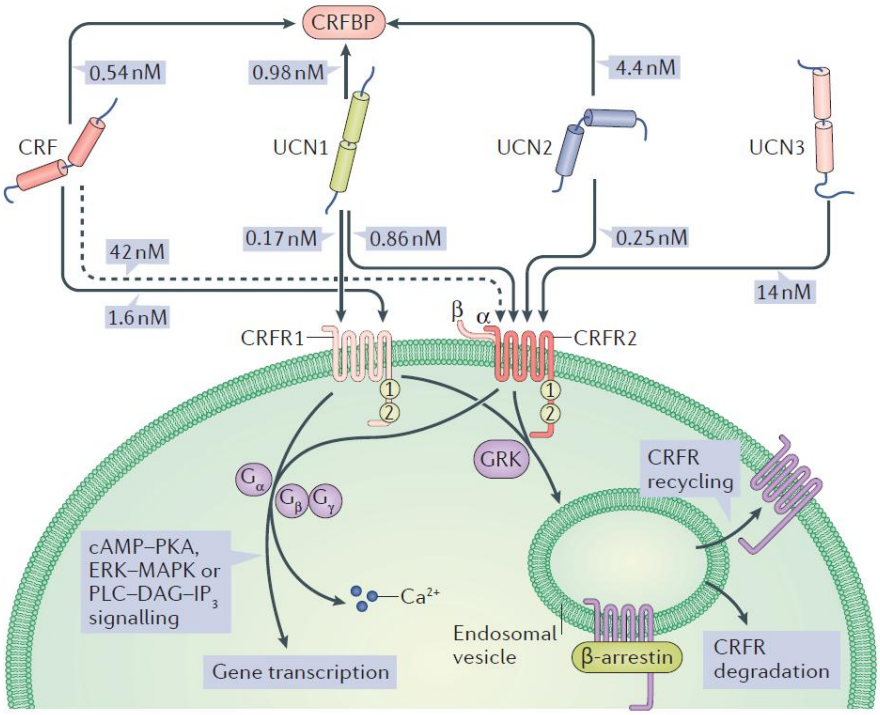

Figure 6. The pharmacological properties and intracellular signalization of the CRFR agonists

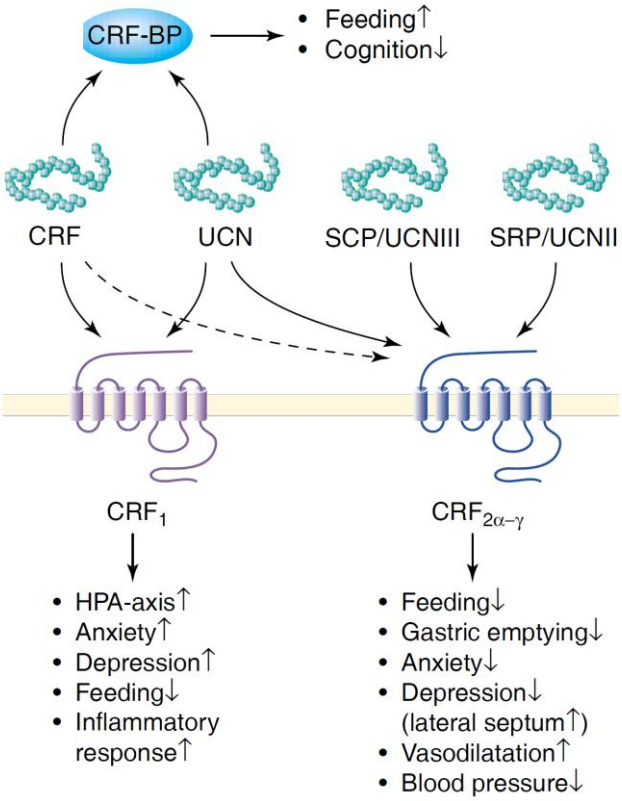

Figure 7. The physiological effects of the CRFR agonists [15]

Central administration of CRF and UCN I induced activation of the HPA axis, anxiety-like and depression-like behavior [1, 4, 16], while central administration of UCN II and UCN III produced anxiolytic and antidepressant actions [34-37]. Accordingly, activation of the CRFR1, expressed predominantly in the cerebral cortex and the anterior pituitary, is believed to initiate the neuroendocrine, autonomic and behavioral reactions to stress [4, 10, 33], whereas activation of the CRFR2, expressed centrally in hypothalamus and the lateral septum, is thought to terminate these stress responses [4, 10, 33] **(Figure 7)**. Nevertheless, the exact role of CRFR1 and CRFR2 in the stress response and stress-related behavior is still under debate [19-20], because studies in mice and rats led to contradictory findings [38-42] **(Figure 8)**.

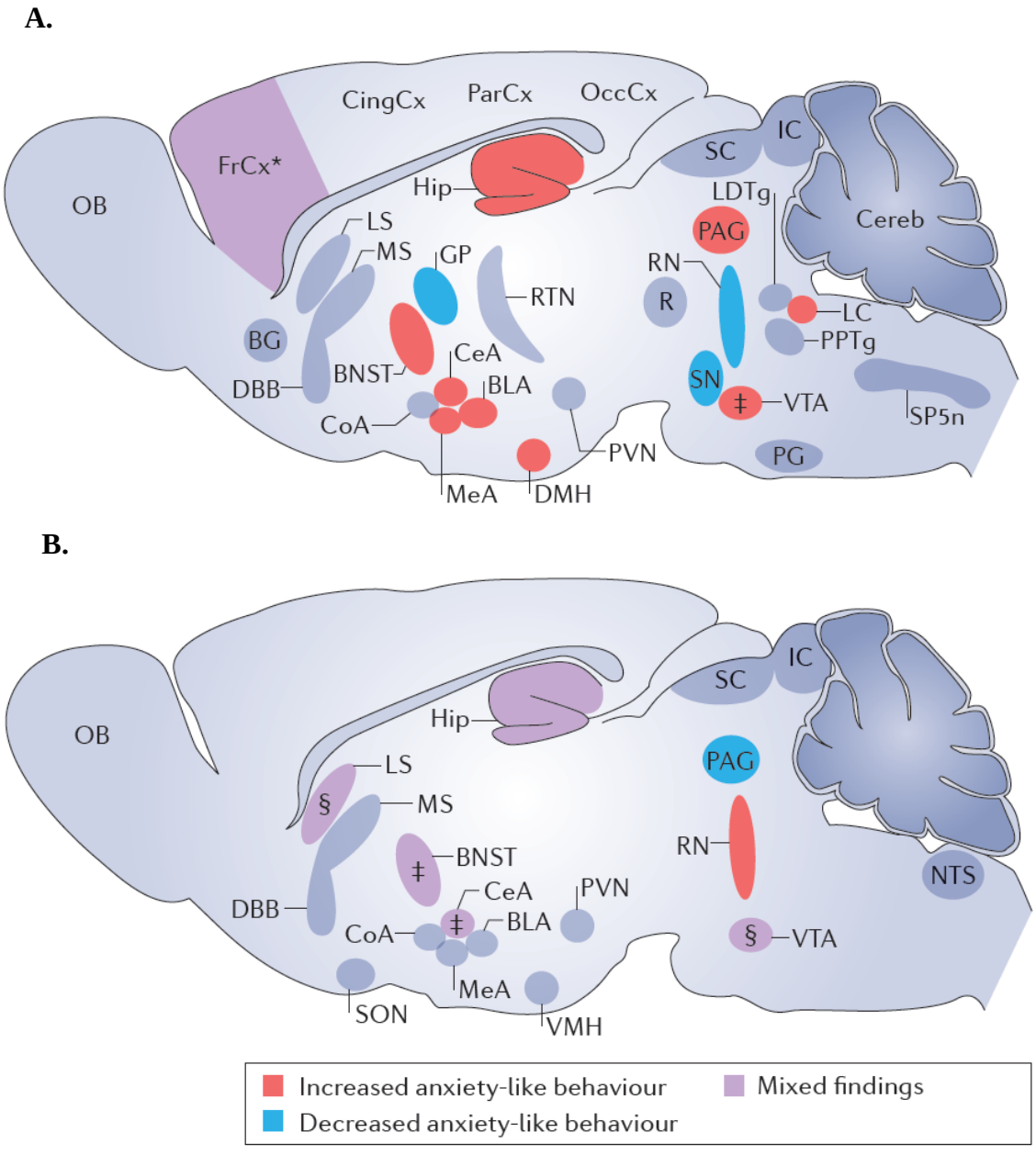

Figure 8. The role of CRFR1 and CRFR2 in the stress-related behavior [15]

1.3. CRF receptor antagonists

In order to determine the role of CRF in the regulation of the neuroendocrine, autonomic and behavioral stress responses, non-selective CRFR antagonists were developed. The first CRF antagonist synthesized was α-helical CRF 9-41, the second was D-Phe CRF; both being peptidic and competitive CRFR antagonists [43-45]. Both anatgonists were derived from CRF and blocked efficiently the CRF- and stress-induced ACTH secretion and locomotor activation [43-45]. The third CRFR antagonist synthesized, astressin was shown to be particularly potent at inhibiting the HPA axis [46]. This antagonist was also proved to reduce the CRF- and stress-induced anxiogenic-like behavior, but it failed to reverse the CRF and stress-induced locomotor hyperactivity [46].

To investigate the exact role of CRFR1 and CRFR2 in the stress responses, selective CRFR antagonists were also developed **(Figure 9)**. The first selective CRFR1 antagonist was CP-154,526 that was followed by its structural analogue antalarmin [47-51]. These are non-peptidic and competitive CRFR1 antagonists, which are able to penetrate the blood-brain barrier and inhibit the stress-induced neuroendocrine and behavioral response [47-51]. Therefore it was suggested that selective CRFR1 antagonists could be used as future therapy in stress-related psychiatric diseases, such as anxiety, depression, post-traumatic stress disorder (PTSD) and panic disorder [33]. The first selective antagonists of CRFR2 were antisauvagine-30 and astressin2B, derived from the frog analogue sauvagine and the non-selective CRF antagonist astressin, respectively [43, 52-55]. These antagonists have peptidic structure, hence they are not able to penetrate the blood-brain barrier. Therefore, they are administered preferentially in the periphery, for example in order to elucidate the role of CRFRs in colonic transit and gastric emyptying [43, 52-55]. The use of selective CRF antagonists concluded that CRFR1 increases colonic transit, whereas CRFR2 decreases gastric empyting, thus it was suggested that selective CRFR2 antagonists could be used as potential therapeutics in stress-related GI disorders, such as in inflammatory bowel diseases (IBD) and irritable bowel syndrome (IBS) [52] **(Figure 10)**.

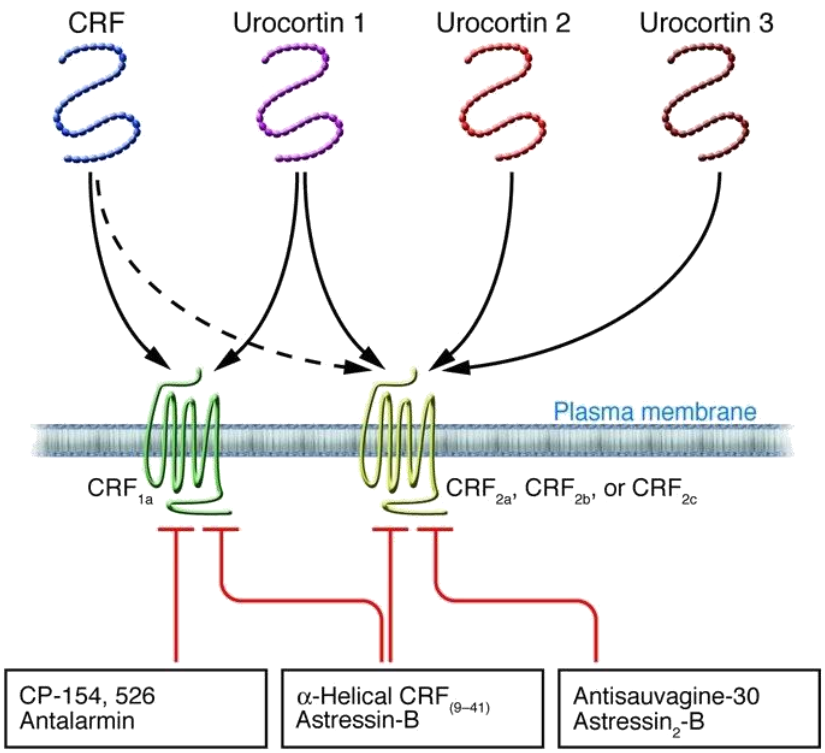

Figure 9. The pharmacological actions of the non-selective and selective CRFR antagonists [56]

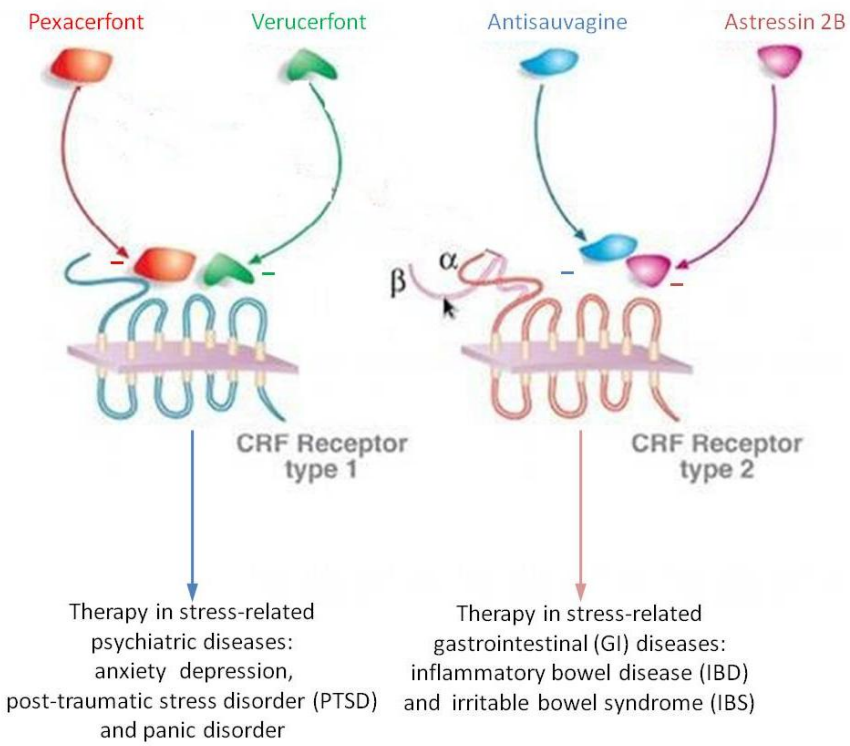

Figure 10. The therapeutical actions of the selective CRFR antagonists [57]

1.4. Nicotine and nicotine receptors

Besides the regulation of the stress responses, CRF has been implicated in nicotine addiction based on several lines of evidence [58-59]. First, acute administration of nicotine, like any other stressor, induces a dose-dependent activation of the HPA axis that is initiated by hypothalamic CRF [58-59]. Second, nicotine withdrawal syndrome resembles the behavioral stress response that is mediated by extrahypothalamic CRF [58-59]. Third, exposure to stressors is one of the leading causes of nicotine relapse that implies the activation of the CRF systems [58-59]. Finally, both CRF receptors seem to participate in the acute, chronic and withdrawal actions of nicotine [60-64]. The basis of nicotine addiction is a combination of positive reinforcement, given by the rewarding, positive effects of nicotine, and negative reinforcement, maintained by the avoidance of the aversive, negative effects of nicotine withdrawal [65] **(Figure 11)**.

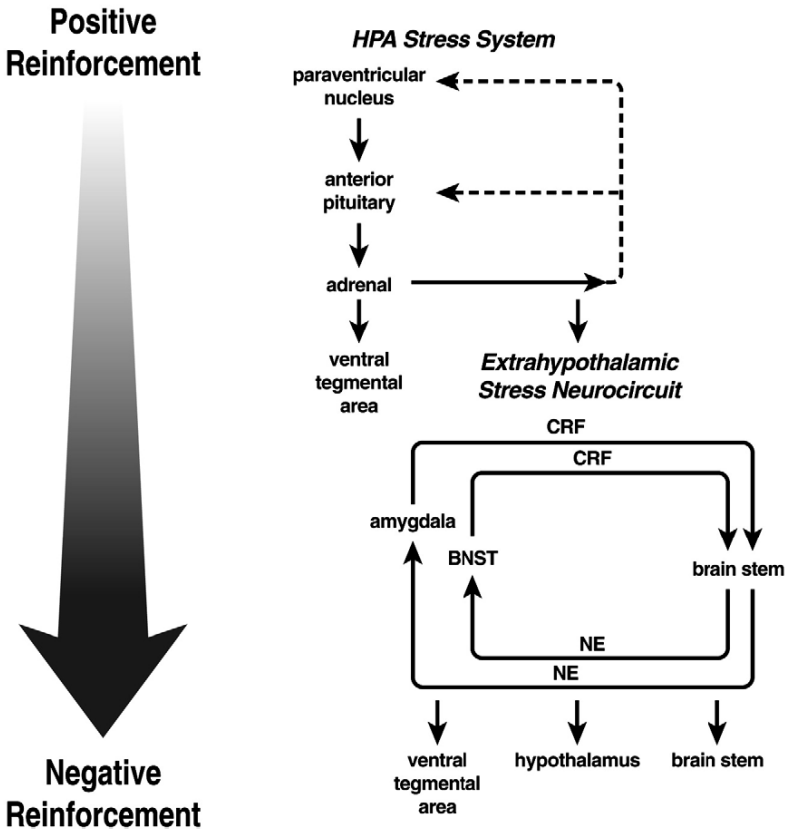

Figure 11. The basis of nicotine addiction

Nicotine is the main psychoactive component of tobacco that causes addiction [66]. Nicotine addiction leads to the harmful habit of smoking that has high morbidity and mortality throughout the world [66]. It is the most frequent type of substance dependence from all substances of abuse resulting in the loss of more than 7 million people per year worldwide [66]. According to the World Health Organization (WHO), in 2018 more than 6 million people died due to first hand smoking, while 1 million were non-smokers, being exposed to second-hand smoking [66]. Nicotine is an alkaloid that is naturally extracted from tobacco; there is approximately 1 mg of nicotine in every cigarette produced from tobacco [66]. During cigarette smoking nicotine enters the lungs, it is absorbed into the bloodstream and reaches the brain in about 8 seconds [66]. Besides its psychostimulant effect, nicotine induces increase of the heart rate, arterial and venous constriction *via* release of adrenaline, but also contraction of the skeletal and smooth muscles *via* release of acetylcholine [66].

The actions of nicotine are mediated by nicotinic acetylcholine receptors (nAChRs) that are considered ligand-gated ion channels composed of pentameric combinations of α and β subunits, since normally they respond to acetycholine [65]. Binding to these pentametric ligand-gated ion channels, nicotine causes a rotation of the receptor that results in the opening of the integral cation channel [65]. Activation of nAChRs leads to increased permeability to both Na^+ and Ca^{2+} resulting in local depolarization, inducing the release of various neurotransmitters [66-67]. Based on their primary sites of expression, nAChRs are classified into two subtypes: muscle-type nicotinic receptors found in neuromuscular junctions and neuronal-type nicotinic receptors found on neuronal bodies and nerve terminals [65]. In the brain there are nine isoforms of the neuronal α-subunit (α2- α10) and three isoform of β-subunit (β2- β4). These are combinations of two α- and three β-, or five α7-subunits with different distinct pharmacological properties, as regards nicotine sensitivity and rate of desensitization [66-67]. The most abundant neuronal nAChRs are α4β2, α3β4 and α7 located both pre- and postsynaptically where they can influence the release of other neurotransmitters, such as dopamine, glutamate and gamma-aminobutyric acid (GABA) [66-67] **(Figure 12)**.

A better understanding of the mechanisms of nicotine addiction has led to the development of new drugs, such as varenicline (a partial agonist of the α4β2 nAChR) and bupropion (an antagonist of nAChRs), which block the negative and positive reinforcement, respectively [65].

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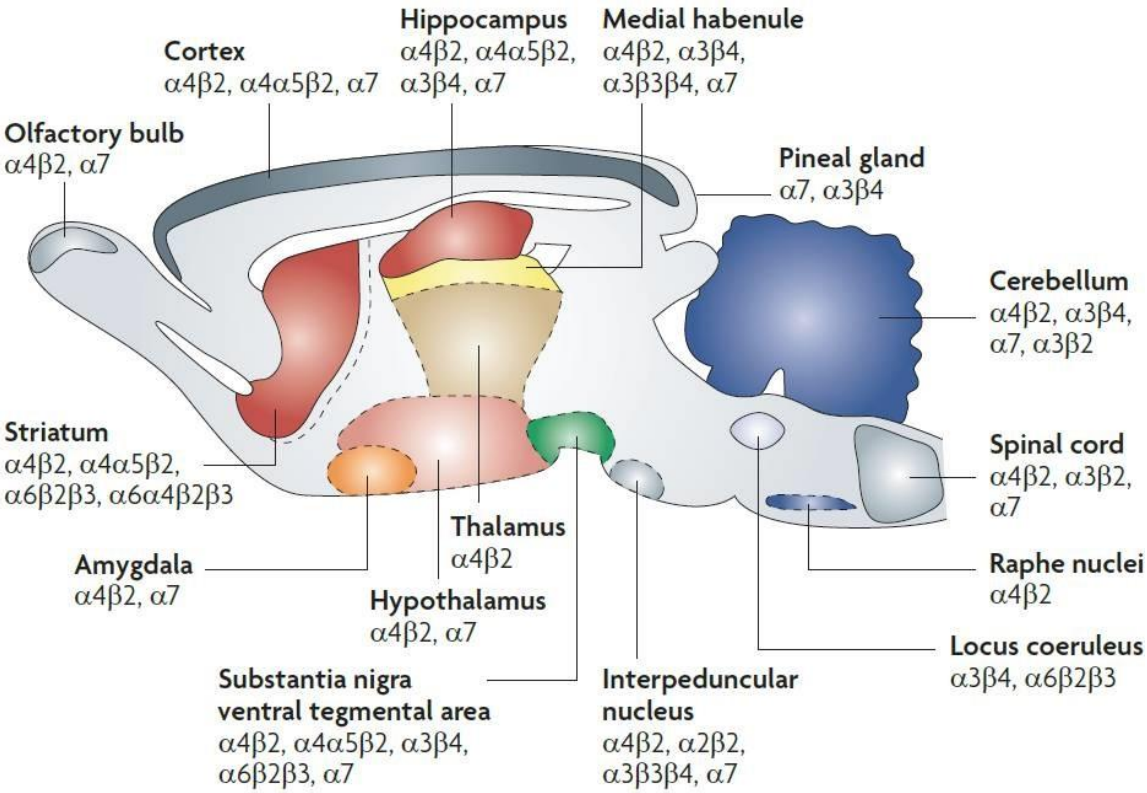

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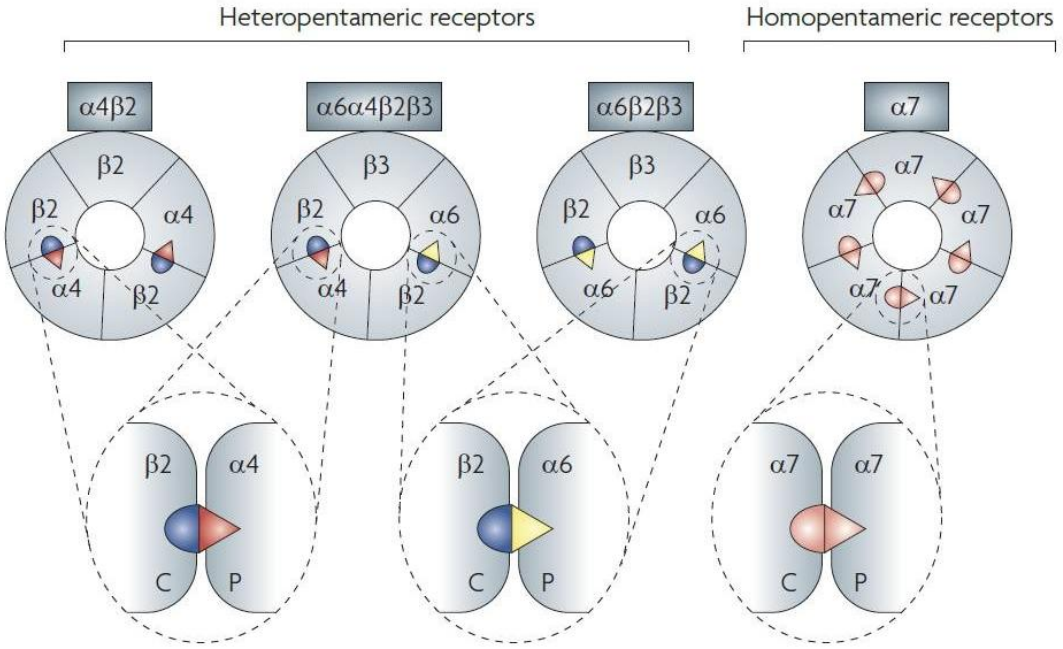


Figure 12. The anatomical distribution **(A)** and chemical structure **(B)** of nAChRs [68]

2. AIM OF STUDY

Besides different types of nAChRs, CRFR1 and CRFR2 can also be potential targets in the therapy of nicotine addiction. Based on the initial concept that CRFR1 and CRFR2 mediate mainly antagonistic effects in the CNS, there are two potential ways to approach the therapy of nicotine addiction: to use selective antagonists of CRFR1, such as antalarmin or to use selective agonists of CRFR2, such as UCN II and UCN III [3-4].

Previous studies have already indicated that blocking CRFR1 would reduce some of the affective symptoms (the dysphoria and the reward deficit) [60, 62-63, 125], whereas blocking CRFR2 would reverse some of the somatic symptoms (the excessive food intake and the increased body weight) of nicotine withdrawal syndrome [64]. Other studies have also indicated that activating CRFR2 would reduce the anxiety- or depression-like behavior observed during alcohol withdrawal [126-127].

The aim of the present study was to investigate the potential therapeutical actions of selective CRFR1 and CRFR2 antagonists and selective CRFR2 agonists in rodents exposed to chronic nicotine treatment and consequent acute nicotine withdrawal. On the one hand, the effects of antalarmin and astressin 2B on the alterations of the dorsal and ventral striatal dopamine release and the vertical and horizontal locomotor activity were examined in nicotine-treated rats. On the other hand, the impacts of UCN II and UCN III upon the anxiety- and depression-like behavior and hyperactivity of the HPA axis were determined in nicotine-treated mice.

3. MATERIALS AND METHODS

3.1. Materials

The selective CRFR agonists used in the experiments were:

UCN II (Bachem, Switzerland), a selective CRFR2 agonist;

UCN III (Bachem, Switzerland), a selective CRFR2 agonist.

The selective CRFR antagonists used in the experiments were:

Antalarmin (Sigma-Aldrich, UK), a selective CRFR1 antagonist;

Astressin 2B (Sigma-Aldrich, UK), a selective CRFR2 antagonist.

Other substances used in experiments were:

[^{3}H]dopamine (Amersham, USA), a tritium-labelled excitatory neurotransmitter;

Krebs solution: NaCl, KCl, $MgSO_4$, $NaHCO_3$, glucose, KH_2PO_4 and $CaCl_2$ (Reanal, Hungary) for incubation and superfusion of the tissues;

Saline solution (NaCl inj. of 0.9 %, Biogal, Hungary);

Nicotine solution (1.4-2 mg/kg, Biogal, Hungary);

Ultima Gold (Perkin Elmer, USA), a scintillation fluid;

Mixture of 5 % CO_2 and 95 % O_2 for continuous gassing of the tissues;

Euthasol (Pentobarbital sodium, CEVA-Phylaxia, Hungary) for general anesthesia of the rats;

Ethyl alcohol, methylene chloride and sulfuric acid of analytical grade (Reanal, Hungary) for determination of the plasma corticosterone concentration;

Ethyl alcohol and sodium hypochlorite (Reanal, Hungary) for cleaning in between the experimental sessions.

3.2. Animals

First, male Wistar rats weighing 150-250 g upon arrival were used (N = 80). 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* [69].

Second, male CFLP mice weighing 24-30 g were used (N = 77). The animals were housed in their home cages at constant room temperature (23°C) on a standard illumination schedule, with 12-h light and 12-h dark periods (lights on from 6:00 a.m.). Commercial food and tap water were available *ad libitum* [70].

The animals were allowed for 7 days to acclimatize before surgery and they were handled daily to minimize the effects of non-specific stress. Although sexually maturized, the experimental animals were considered adolescents, since they were about 6-7 weeks old when the experiments had started [71]. Pre-adolescence and adolescence are developmental periods associated with increased vulnerability for nicotine addiction, and exposure to nicotine during these periods may lead to long-lasting changes in behavioral and neuronal plasticity in different brain regions, such as the cerebral cortex, hippocampus and striatum [72-73]. During the experiments the animals were kept and handled in accordance with the instructions of the University of Szeged Ethical Comittee for the Protection of Animals in Research.

3.3. Surgery

The rats were implanted with a stainless steel Luer cannula (10 mm long), aimed at the right lateral cerebral ventricle under anesthesia with 60 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 the stereotaxic atlas of the rat brain [74]. Cannulas were secured to the skull with dental cement and acrylate. The rats were allowed for 7 days to recover before experiments were started. After the experiments were concluded, 10 μl of methylene blue at 1 g/100 ml were injected into the lateral cerebral ventricle of the decapitated animals and the position of the cannula was inspected visually. Animals without the dye in the lateral cerebral ventricle (8 from 80) were excluded from the final statistical analysis.

The mice were implanted with a polyethylene Luer cannula (6 mm long) aimed at the right lateral cerebral ventricle under anesthesia with 60 mg/kg of pentobarbital sodium. The stereotaxic coordinates were 0.5 mm posterior and 0.5 mm lateral to the bregma, and 3 mm deep from the dural surface [75]. Cannulas were secured to the skull with cyanoacrylate containing instant glue. The mice were allowed for 7 days to recover after the surgery. After the experiments were ended, 2 μl of methylene blue was injected into the lateral cerebral ventricle of the decapitated animals to check the permeability and the right position of the cannula. Animals without the dye in the lateral cerebral ventricle (5 from 77) were omitted from the final statistical analysis.

3.4. Treatment

The rats were treated intraperitoneally (IP) with 1.4 mg/kg nicotine tartrate or 10 ml/kg of 0.9% saline solution for 7 days, two times per day (at 8:00 and at 20:00).

Half of the rats were treated intracerebroventricularly (ICV) with 0.1 μg/2μl antalarmin or 1 μg/2μl astressin 2B or 2 μl of 0.9% saline solution on the 8th day (after 12 hours following the last IP administration). The other half of the animals were treated ICV on the 9th day (after 24 hours following the last IP administration) based on the same treatment protocol. Hence, rats were divided in 6 groups: group 1. saline IP + saline ICV; group 2. saline IP + antalarmin ICV; group 3. saline IP + astressin 2B ICV; group 4. nicotine IP + saline ICV; group 5. nicotine IP + antalarmin ICV; and group 6. nicotine IP + astressin 2B ICV [69].

The mice were treated IP with 2 mg/kg nicotine tartrate or 10 ml/kg saline solution for control for 7 days, 4 times per day. Half of the mice were treated ICV on the 8th day (12 hours after the last IP treatment), the other half on the 9th day (24 hours after the last IP treatment) with 2 μg/2μl UCN II, 2 μg/2μl UCN III or 2 μl of saline solution for control. Thus, mice were divided in 6 groups based on the following treatments: 1. saline IP + saline ICV, 2. saline IP + UCN II ICV, 3. saline IP + UCN III ICV, 4. nicotine IP + saline ICV, 5. nicotine IP + UCN II ICV and 6. nicotine IP + UCN III ICV [70].

The doses of nicotine and the schedule of administration chosen were expected to produce plasma nicotine levels in rats similar to plasma nicotine levels found in an individual who smokes 1-2 packs of cigarettes per day [76].

3.5. *In vivo* conducta studies

Thirty minutes after the ICV injection, the horizontal and vertical locomotor activities were recorded in an *in vivo* conducta system (MDE, Ltd, Germany), which is based on the principles of the open-field test and was described in previous studies [77-78]. The apparatus was a square open-field black box with a side length of 60 cm, surrounded by a 40 cm high wall. The floor of the box was divided in 36 (6×6) small squares. Five by five rows of photocell beams allowed a computer-based system to register the behavioral activity of each animal. A 60 W light was situated 1 m above the arena floor. Each animal was carried to the experimental room in their home cage and placed in the center of the box with which they were familiarized for 5 min. Then the horizontal activity, representing a measure of overall activity and arousal, and the vertical activity, representing a measure of exploratory and stereotype behavior, were monitorized for 30 minutes. The box was cleaned between sessions with 96 % ethyl-alcohol.

3.6. *In vitro* superfusion studies

After the decapitation of the rats the changes of the dorsal and ventral striatal dopamine release were determined by an *in vitro* superfusion system (MDE, Ltd, Germany) described in previous studies [79-82]. The striatum was isolated and dissected in a Petri dish filled with ice-cold Krebs solution. The stereotaxic coordinates were 4.0 mm anterior and 1.0 mm posterior to the bregma, according to the stereotaxic atlas of the rat brain [74]. The dorsal striatum, including the putamen and the nucleus caudatus, and the ventral striatum, including the nucleus accumbens (NAcc), were decapsulated from the surrounding white matter and separated from each other. Slices of 300 μM were produced with a McIlwain tissue chopper (Campden Instruments Ltd., UK). The slices 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% CO_2 and 95% O_2. During the incubation, the slices were labelled with 15 μmol of [^{3}H]dopamine with a specific activity of 14 Ci/mmol. Two tritiated slices were transferred to each of the four cylindrical perspex chambers of the superfusion system. Gold electrodes were attached to both halves of the superfusion chambers and connected to an ST-02 electrical stimulator (MDE, Co. Ltd., Germany). A multichannel peristaltic Gilson Minipuls 2 pump (Gilson Inc., USA) was used to maintain a constant superfusion rate of 300 μl/min. The slices were superfused for 30 min to allow tissue equilibrium, and the superfusates were collected in Eppendorf tubes by a multichannel fraction collector Gilson FC 203B (Gilson Inc., USA). After 2 minutes electrical stimulation consisting of square-wave impulses (intensity: 10 mA, voltage: 100 V, pulse length: 5 ms, frequency: 10 Hz) was delivered to each of the four chambers lasting two minutes. The sample collecting lasted 32 minutes (2 minutes for each sample) and the peak of the fractional release was observed at 14 minutes. The remnants of slices were solubilized in 200 ml of Krebs solution, using an ultrasonic homogenizer Branson Sonifier 250 (Labequip Ltd., Canada). The radioactivity in the fractions and the homogenized tissue samples was measured with a liquid scintillation spectrometer (Tri-carb 2100TR, Packard Inc., USA) after the addition of 3 ml of scintillation fluid. The fractional release was calculated as a percentage of the radioactivity present in each collected sample compared to the total radioactivity of the correspondent tissue.

3.7. Elevated plus-maze test

Thirty minutes after the ICV treatment, the animals were evaluated in an elevated plus-maze test, validated by Lister and Rodgers to investigate anxiety-like behavior [83-84]. The 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 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 the following parameters were recorded by an observer sitting at 100 cm distance from the center of the plus-maze: a. the percentage of the number of entries into the open arms relative to the total number of entries, b. the percentage of the time spent in the open arms relative to the total time and c. the total number of entries into the open and the closed arms. Entry into an arm was defined as the entry of all four feet of the animal into that arm. The apparatus was cleaned up with sodium hypochlorite solution between the subjects.

3.8. Forced swim test

In parallel, the animals were evaluated in a forced swim test, invented by Porsolt et al. to investigate depression-like behavior [85]. The 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 dropped 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: a. the climbing activity (the time that animals spent with climbing the walls, in their attempt to escape the cylinder), b. the swimming activity (the time that animals spent with swimming in the water, in their attempt to remain at the surface) and c. the time of immobility (the time that animals spent in an upright position on the surface with its front paws together). A 5 second period was considered a time unit, thus the climbing and the swimming activities and the time of immobility were expressed in time units. The water from the apparatus was changed between the subjects.

3.9. Chemo-fluorescent assay

After the decapitation of the mice, the trunk blood was collected for determination of plasma corticosterone concentration by a chemo-fluorescent assay that was described by Zenker and Bernstein and later modified by Purves and Sirett [86-87]. According to this method, the trunk blood was collected into heparinized tubes and centrifuged for 10 min at 3000 rpm for determination of the plasma corticosterone levels. Two hundred ml aliquots of the medium were transferred to centrifuge tubes. A reagent blank of 200 µl of distilled water and 2 corticosterone 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 tube and rocked for 30 min to allow complete extraction of corticosterone by the solvent. The extract is centrifuged for 10 min at 3000 rpm. In order 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. Five 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 min, centrifuged at 3000 rpm for 10 min and was allowed to stand at room temperature for 2 hours, which permitted the maximum development of fluorescence from corticosterone. Emission intensity was measured from the lower sulfuric acid layer with Hitachi 204-A fluorescent spectrophotometer at 456 nm extinction and 515 emission wave-length. The concentration of corticosterone of the samples was calculated from the values of the standards and expressed in µg/100 ml.

3.10. Statistical analysis

Statistical analysis of the results was performed by analysis of variance (Prism 7 Statistics, GraphPad Inc., USA). The differences between groups were determined by two-way ANOVA followed by Tukey’s test for pairwise comparisons when prerequisites were fulfilled. A probability level of 0.05 or less was accepted as indicating a statistically significant difference.

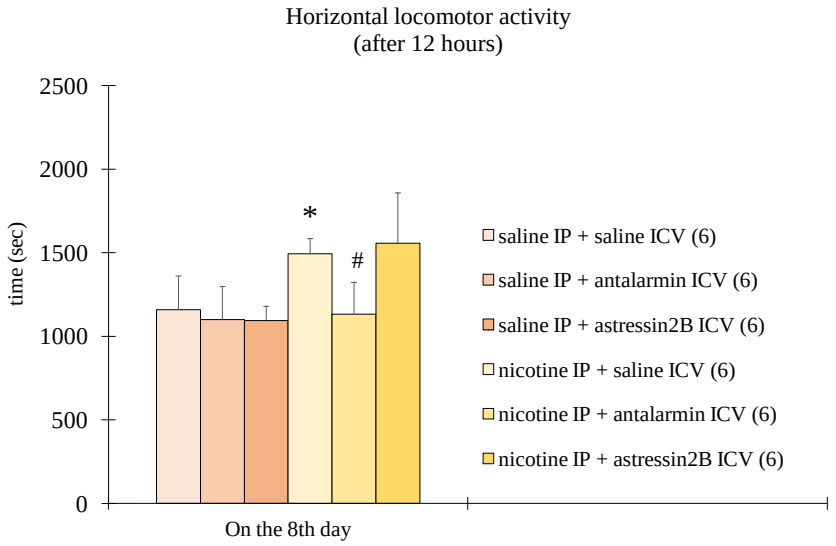
4. RESULTS

4.1. After 12 hours of nicotine withdrawal

On the 8th day, the horizontal and vertical locomotor activity increased significantly in nicotine-treated rats, compared with the saline-treated rats **(Figure 13)**. In parallel, the dorsal and ventral striatal dopamine release increased significantly in nicotine-treated group, compared with the saline-treated group **(Figure 14)**. All the changes observed on the 8th day were reduced significantly after treatment with antalarmin, but not astressin 2B [69] **(Figures 13-14)**.

On the 8th day, the time spent in the open arms and the number of entries into the open arms increased remarakably, with the first parameter increasing significantly, but the total number of entries did not change significantly in nicotine-treated mice, compared to the saline-treated ones. The first two parameters increased further after treatment with UCN II or UCN III in the nicotine-treated groups, but only the time spent in the open arms was increased significantly **(Figure 15)**. The swimming and the climbing activity decreased significantly in nicotine-treated mice, compared to the saline-treated ones, but the time spent immobile was not altered. After treatment with UCN II and UCN III the swimming and the climbing activity decreased further in the nicotine-treated groups, both parameters decreasing significantly, but the time of immobilization was not altered **(Figure 15)**. The plasma corticosterone concentration was elevated considerably, but insignificantly in the nicotine-treated group, compared to the saline-treated one, and this elevation of the plasma corticosterone level was reduced significantly after treatment with UCN II and UCN III [70] **(Figure 16)**.

A.



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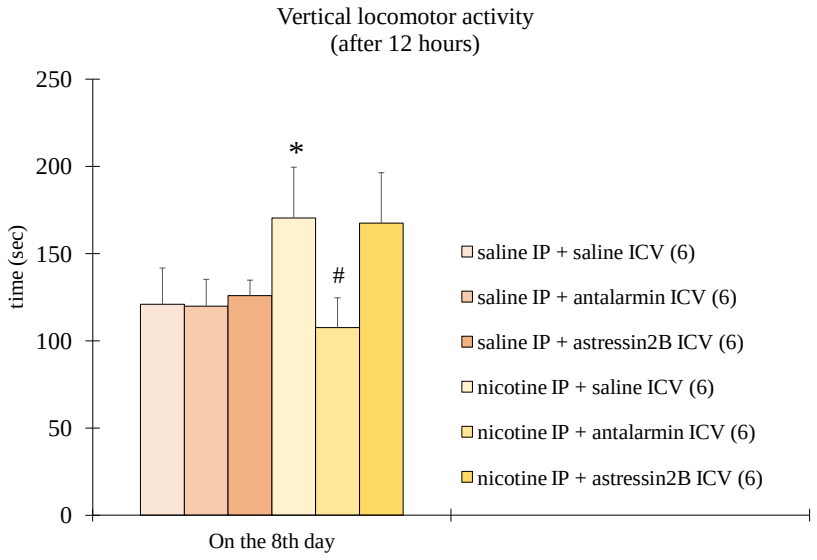
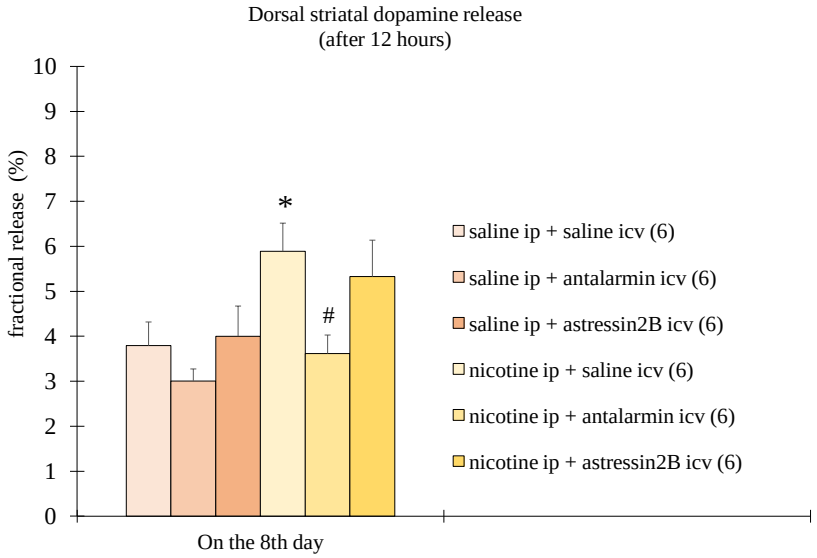


Figure 13. The changes of horizontal **(A)** and vertical locomotor activity **(B)** observed after 12 hours following the last IP administration of nicotine or saline

Statistically significant difference was accepted for $p < 0.05$ and indicated with * for nicotine IP + saline ICV *vs.* saline IP + saline ICV and with # for nicotine IP + antalarmin ICV *vs.* nicotine IP + saline ICV

A.



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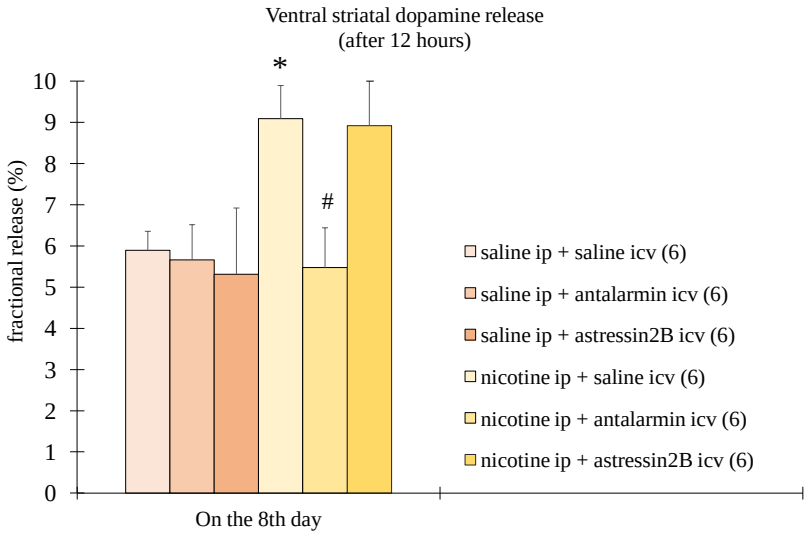
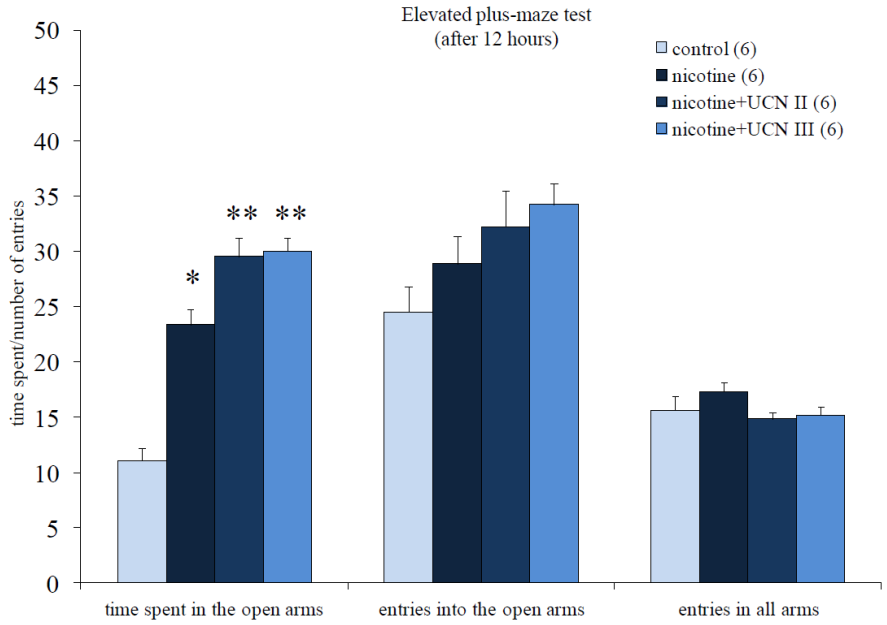


Figure 14. The changes of dorsal **(A)** and ventral striatal dopamine release **(B)** observed after 12 hours following the last IP administration of nicotine or saline

Statistically significant difference was accepted for $p < 0.05$ and indicated with * for nicotine IP + saline ICV *vs.* saline IP + saline ICV and with # for nicotine IP + antalarmin ICV *vs.* nicotine IP + saline ICV

A.



B.

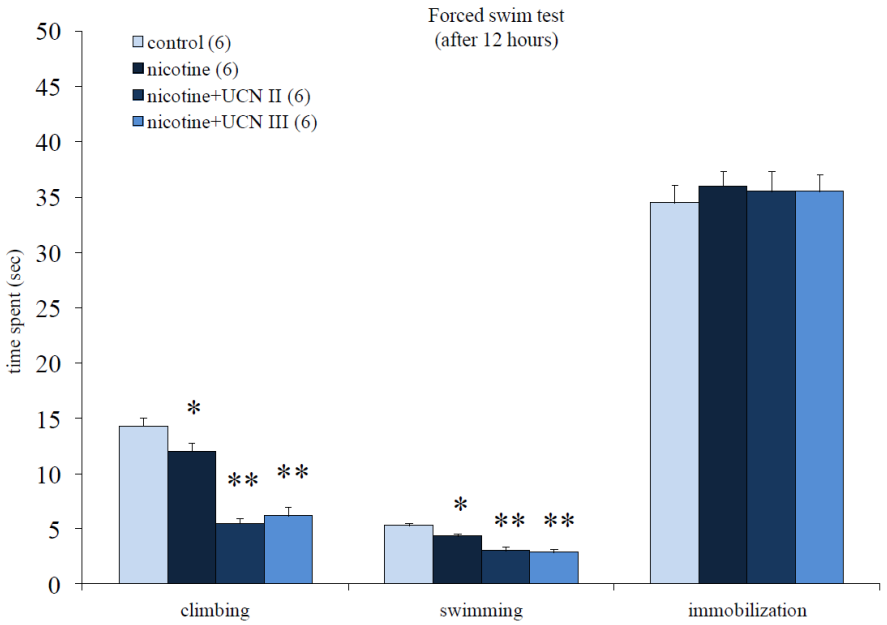


Figure 15. The signs of anxiety **(A)** and depression **(B)** observed after 12 hours following the last IP administration of nicotine or saline

Statistically significant difference was accepted for $p < 0.05$

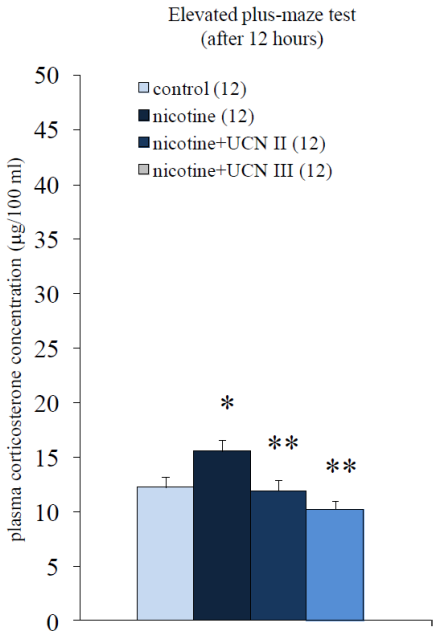


Figure 16. The changes of plasma corticosterone concentration measured after 12 hours following the last IP administration of nicotine or saline

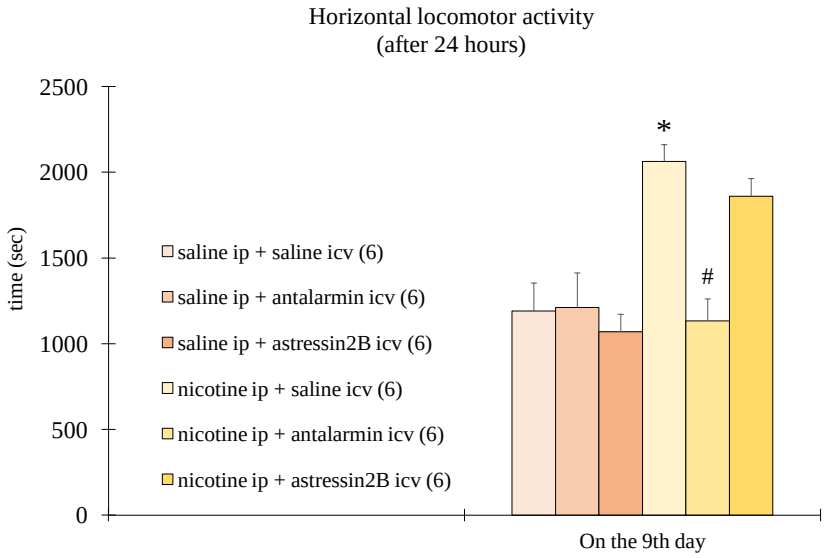
Statistically significant difference was accepted for $p < 0.05$

4.2. After 24 hours of nicotine withdrawal

On the 9th day, significant increases of the horizontal locomotor activity and dorsal striatal dopamine release were observed in the nicotine-treated group, compared with the saline-treated group **(Figures 17-18)**. In contrast, the vertical locomotor activity and the ventral striatal dopamine release were decreased significantly in the nicotine-treated rats, compared with the saline-treated rats **(Figures 17-18)**. All the changes described on the 9th day were reversed completely after treatment with antalarmin, but not astressin 2B [69] **(Figures 17-18)**.

On the 9th day, the number of entries into the open arms and the time spent in the open arms decreased significantly in nicotine-treated mice, compared to the saline-treated ones, but the total number of entries was not affected significantly in the nicotine-treated group. The decreasing effects were inverted after treatment with UCN II and UCN III, but only the number of entries was increased significantly, and the total number of entries was not affected in the nicotine-treated groups **(Figure 19)**. The swimming and the climbing activity decreased significantly, and the time of immobilization increased significantly as well, in nicotine-treated mice, compared to the saline-treated ones. After treatment with UCN II or UCN III the time spent with climbing and swimming was enhanced significantly and the time spent immobile was reduced significantly in the nicotine-treated groups **(Figure 19)**. The plasma corticosterone concentration was augmented remarkably and significantly in the nicotine-treated group, compared to the saline-treated one, but this augmentation of the plasma corticosterone level was abolished completely after treatment with UCN II and UCN III [70] **(Figure 20)**.

A.



B.

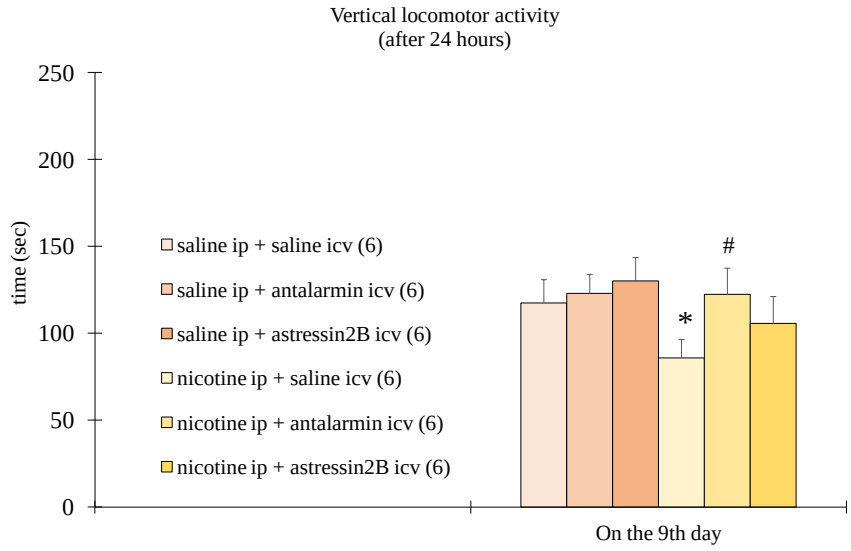
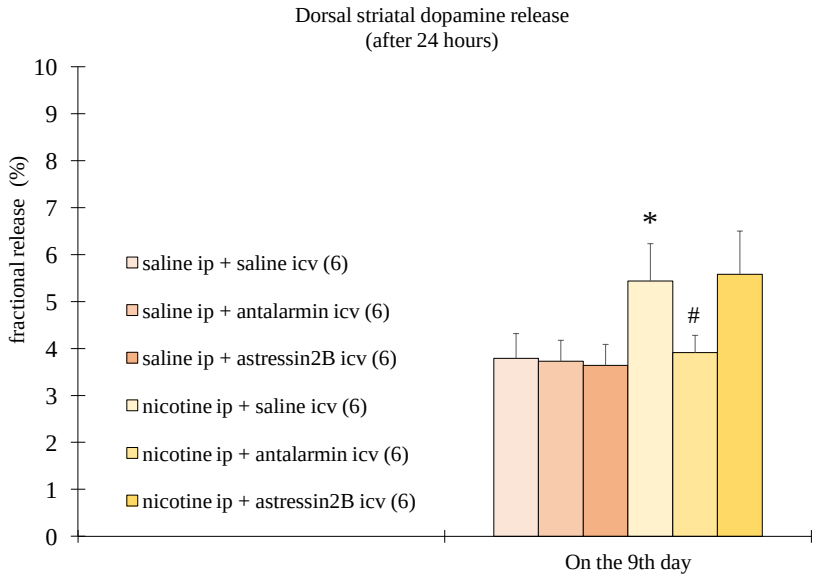


Figure 17. The changes of horizontal **(A)** and vertical locomotor activity **(B)** observed after 24 hours following the last IP administration of nicotine or saline

Statistically significant difference was accepted for $p < 0.05$ and indicated with * for nicotine IP + saline ICV *vs.* saline IP + saline ICV and with # for nicotine IP + antalarmin ICV *vs.* nicotine IP + saline ICV

A.



B.

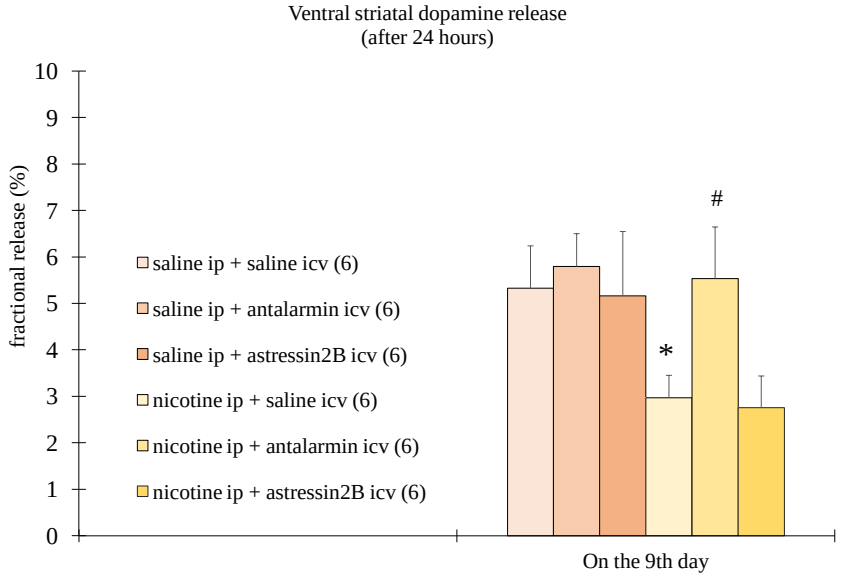
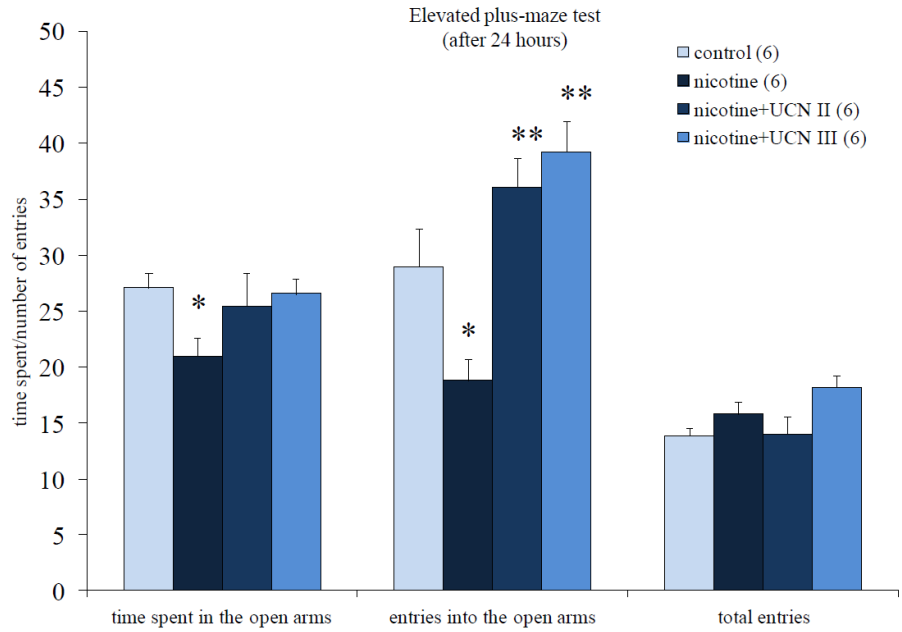


Figure 18. The changes of dorsal **(A)** and ventral striatal dopamine release **(B)** observed after 24 hours following the last IP administration of nicotine or saline

Statistically significant difference was accepted for $p < 0.05$ and indicated with * for nicotine IP + saline ICV *vs.* saline IP + saline ICV and with # for nicotine IP + antalarmin ICV *vs.* nicotine IP + saline ICV

A.



B.

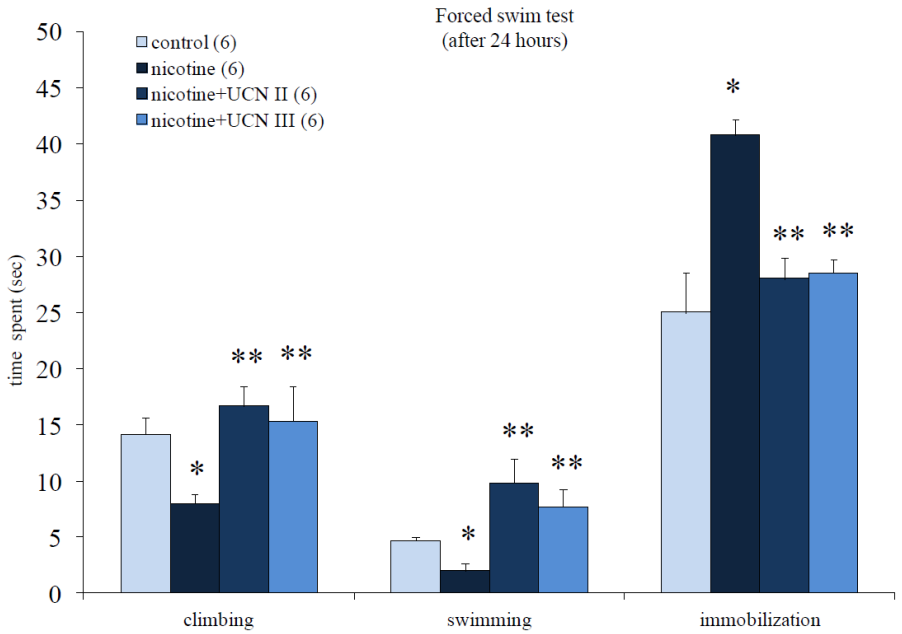


Figure 19. The signs of anxiety **(A)** and depression **(B)** observed after 24 hours following the last IP administration of nicotine or saline

Statistically significant difference was accepted for $p < 0.05$

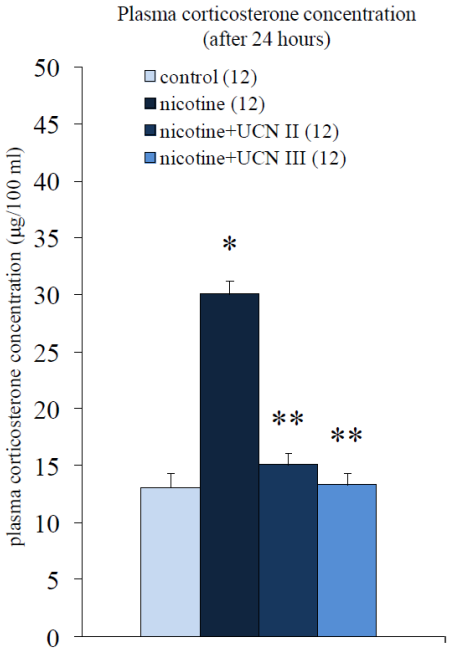


Figure 20. The changes of plasma corticosterone concentration measured after 24 hours following the last IP administration of nicotine or saline

Statistically significant difference was accepted for $p < 0.05$

5. DISCUSSION

5.1. Nicotine addiction: definition

Drug addiction, also known as substance dependence, 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 [88]. According to the latest, fifth edition of Diagnostic and Statistical Manual of Mental Disorders (DSM-5), nicotine addiction or nicotine dependence is described as ”tobacco use disorder” [89]. DSM-5 defines 3 criteria with 15 sub-features and 4 specifiers to set up the clinical diagnosis of tobacco use disorder [90]. For the positive diagnosis one must use tobacco for over one year and at least two of the sub-features should be met [90]. Drug addiction (including nicotine addiction) has been further conceptualized as a dynamic, evolving disorder that consists of three stages: binge/intoxication, withdrawal/negative affect and preoccupation/anticipation or craving [88] **(Figure 21)**.

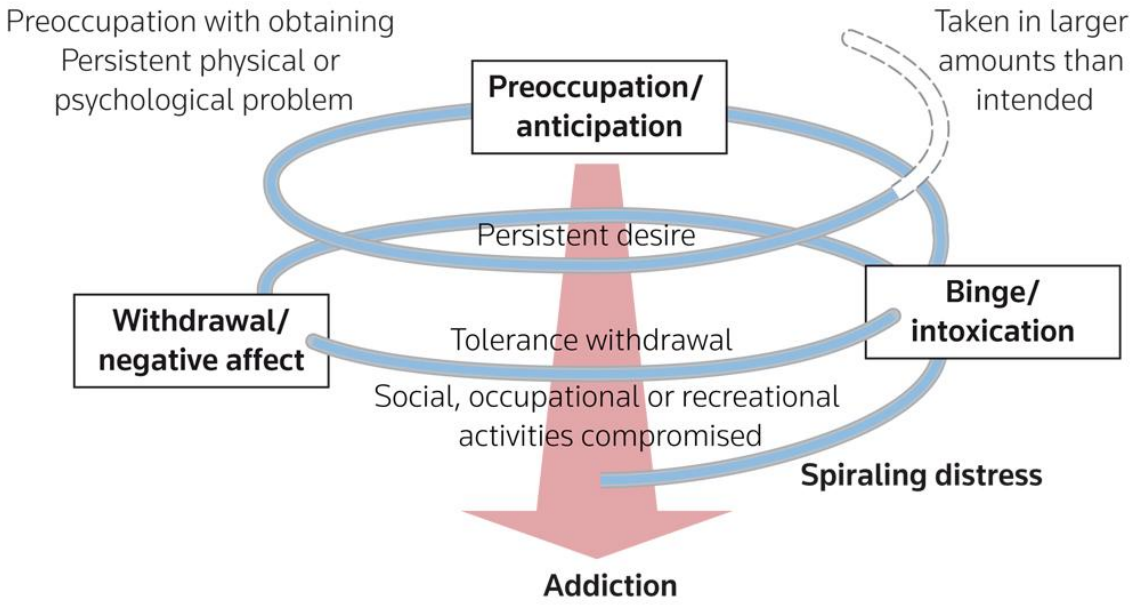


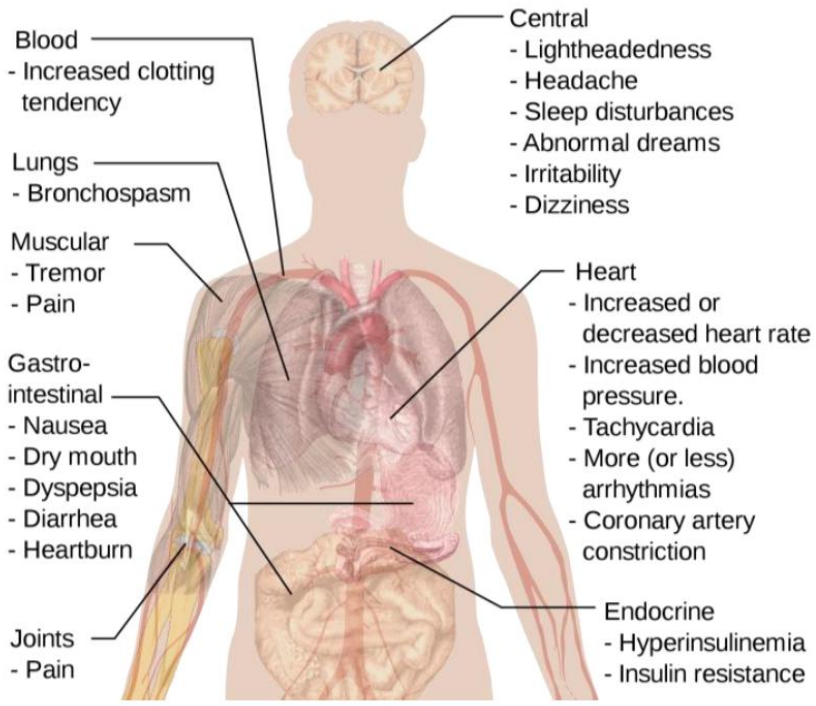
Figure 21. The stages of nicotine addiction [9]

5.2. Nicotine addiction: binge/intoxication

Acute use of nicotine increases the release of the striatal dopamine that is associated with a sensation of reward, euphoria and locomotor hyperactivity in rats [91-92]. The search for the sensation of reward and euphoria represents the positive reinforcement that leads to nicotine addiction [93]. Chronic use of nicotine also increases the striatal dopamine release, although its impact on the locomotor activity depends upon the dose of the drug and the schedule of administration [94-95]. Usually, continuous infusion of nicotine several times a day may induce tolerance, while repetitive injection once a day may produce sensitization to the effects of nicotine [96-97]. Nicotine also augments glutamate release, which stimulates the release of dopamine, and GABA release, which inhibits the release of dopamine. With long-term exposure to nicotine, some nAChRs become desensitized, but some do not [65, 76]. As a result, GABAergic inhibitory tone diminishes, while glutamatergic excitation continues, thereby increasing excitation of dopaminergic neurons and enhancing responsiveness to nicotine [65, 76] **(Figure 22)**.

This stage is mediated by the nigrostriatal and mesolimbic pathways [96-97]. The nigrostriatal pathway originates in the dopaminergic neurons located in the substantia nigra and terminates in the putamen and nucleus caudatus which together constitute the dorsal striatum [96-97]. The mesolimbic pathway arises from the dopaminergic neurons situated in the ventral tegmental area and projects to the NAcc that represents the ventral striatum [96-97]. Classically, the nigrostriatal pathway controls motor behavior, posture and learning of motor programs and habits, whereas the mesolimbic pathway contributes to motor behavior by mediation of reward, emotion and motivation [98]. Nevertheless, manipulations of dopamine release in the dorsal and ventral striatum affect motor behavior in distinct, but parallel ways, which depend upon the nature of the cortical and limbic input to these brain structures [98] **(Figure 24)**.

A.



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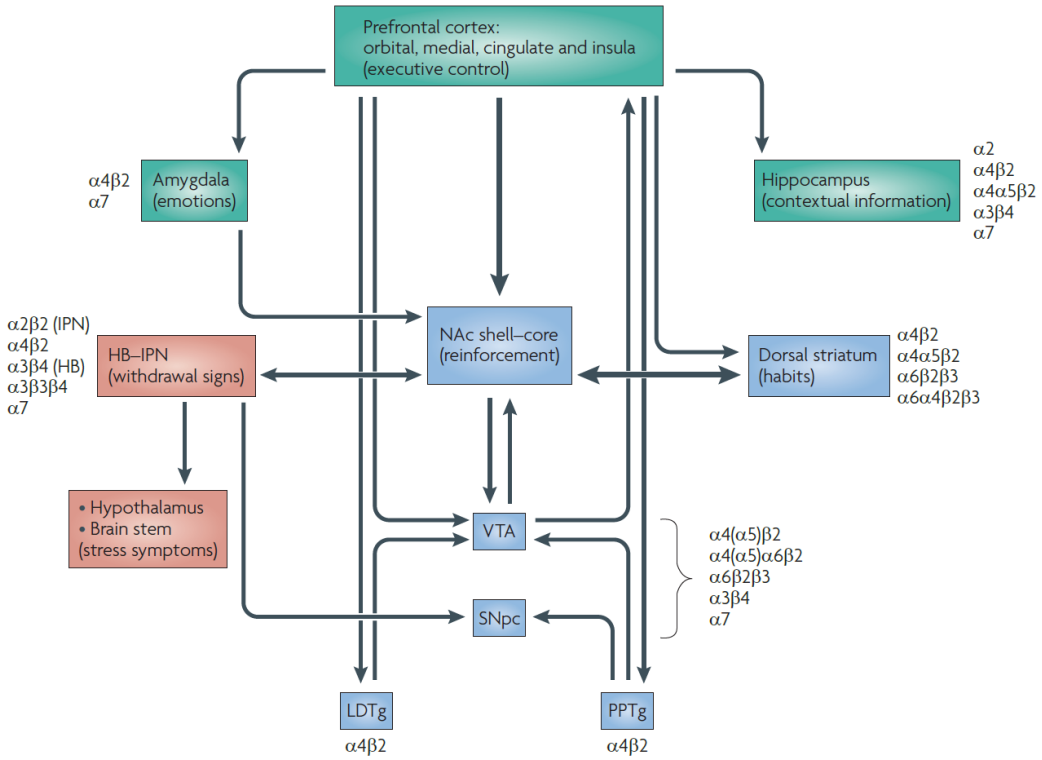
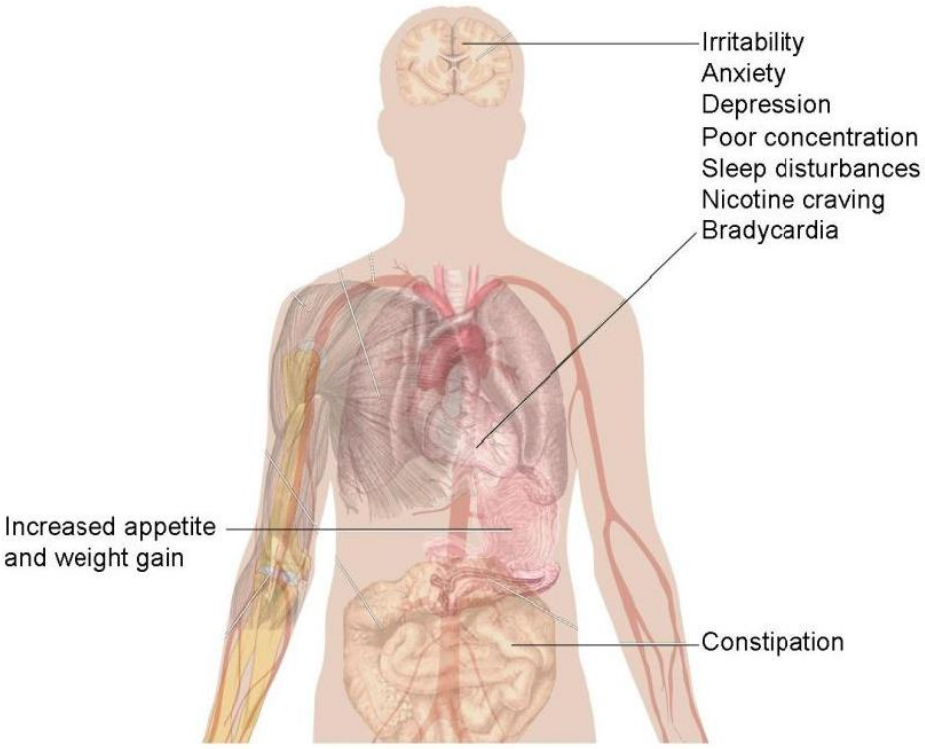


Figure 22. Clinical signs **(A)** and anatomical/pharmacological backround **(B)** of chronic nicotine use [99]

A.



B.

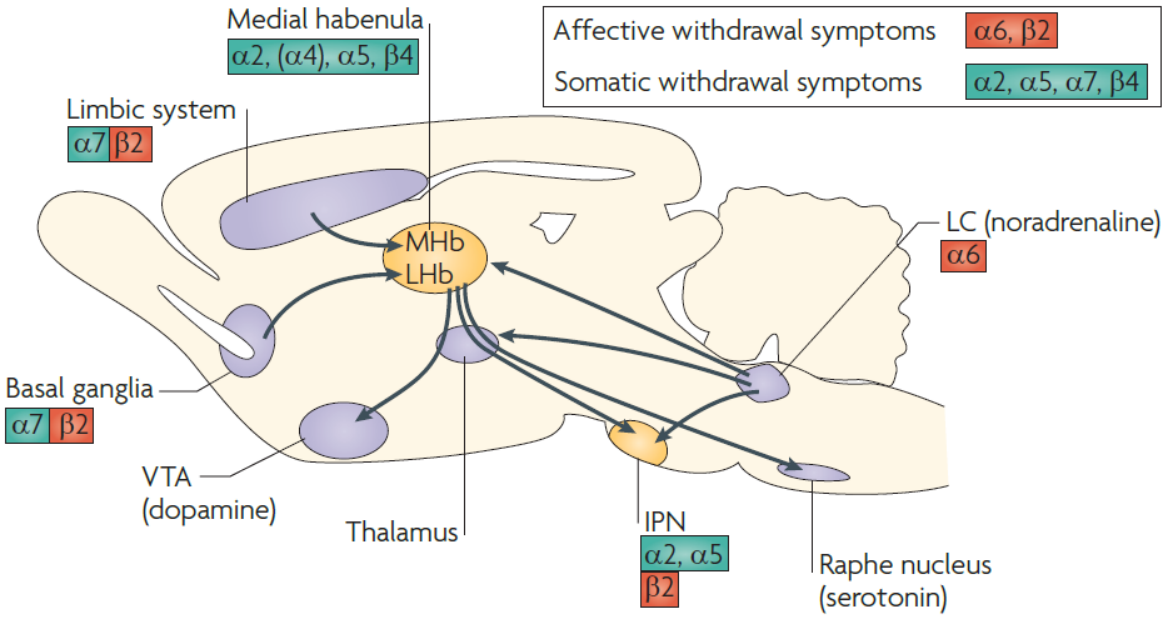


Figure 23. Clinical signs **(A)** and anatomical/pharmacological backround **(B)** of acute nicotine withdrawal [99]

5.3. Nicotine addiction: withdrawal/negative affect

Acute withdrawal following chronic administration of nicotine causes a nicotine withdrawal syndrome that starts promptly within few hours, peaks around 24 hours, and lasts a few days following cessation of chronic nicotine administration [100]. The nicotine withdrawal syndrome in rats consists of a somatic component, characterized by locomotor hypoactivity, increased appetite and weight gain and an affective component, represented by anxiety, depression and reward deficit [101]. The somatic symptoms in humans include bradycardia, gastrointestinal discomfort and increased appetite; in rodents they correspond to locomotor hypoactivity, increased appetite and weight gain and stereotype behavior [66-67, 101]. The affective symptoms in humans incorporate dysphoria, anxiety, depression, irritability and difficulty concentrating; in rodents they correlate with anhedonia, anxiety- and depression-like behavior [66-67, 101] **(Figure 23)**.

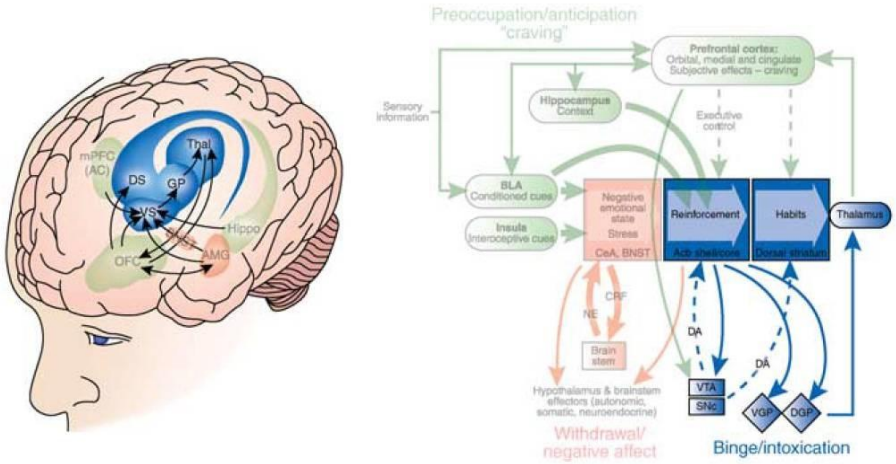
This stage is mediated by the extended amygdala, a functional unit that consists of three anatomically distinct structures: the CeA, the BNST and the shell part of NAcc [93]. The extended amygdala can be regarded as an interface between stress systems and reward systems, the activation of which produces a negative emotional state that is mediated by CRF, NA and dynorphin [93]. The avoidance of the dysphoria, anxiety and depression represents the negative reinforcement that maintains nicotine addiction [93] **(Figure 24)**.

5.4. Nicotine addiction: preoccupation/anticipation or craving

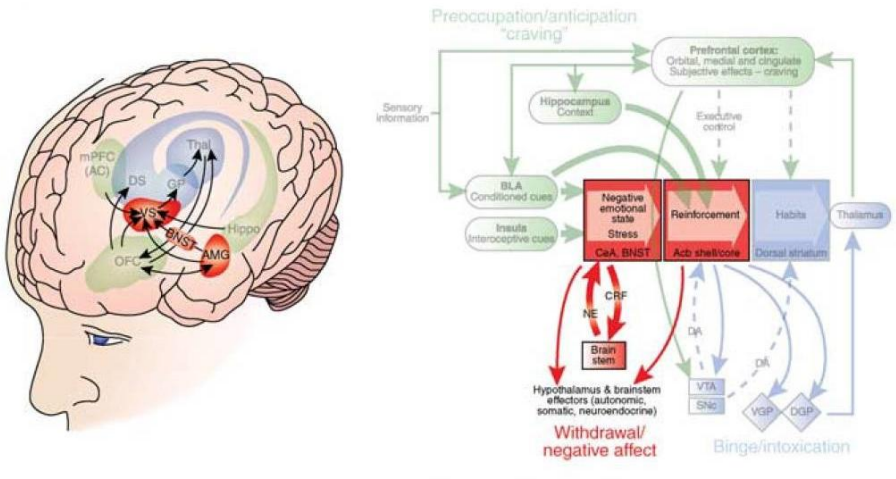
Some of the affective symptoms, such as anxiety and depression, may persist during chronic nicotine withdrawal leading to the third stage of nicotine addiction, characterized by preoccupation/anticipation or craving [93]. The craving for drug use is hypothesized to play a key element in relapse in humans, thus leading to the so called “chronic relapsing disorder” [93]. Exposure to stressors is one of the leading causes of nicotine relapse that implicates the activation of the anti-reward and stress systems for months or years after quitting the drug use [58-59]. Nevertheless, the senzation of craving does not always correlate with relapse and is difficult to measure clinically [93].

The most important brain regions involved in this stage are the basolateral amygdala and the hippocampus which mediate the conditioned reinforcement and processing of contextual information, respectively [93]. The executive control depends on the prefrontal cortex which integrates the signals received from the orbital, medial and cingulate cortex which, in turn, mediate the subjective feeling of craving itself [93]. The major neurotransmitter involved in this stage is glutamate [93] **(Figure 24)**.

A.



B.



C.

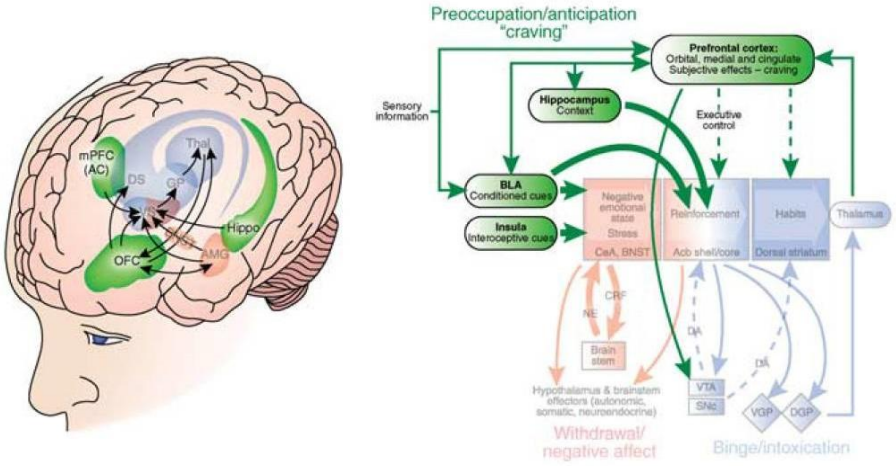


Figure 24. Clinical stages of nicotine addiction: binge/intoxication **(A)**, withdrawal/negative affect **(B)** and preoccupation/anticipation or craving **(C)** [93]

5.5. The effects of chronic nicotine treatment

In concordance with the features of the stage of binge/intoxication, in rats exposed to 7 days of nicotine treatment (after 12 hours following the last nicotine administration) we observed increases in horizontal and vertical locomotor activity along with increases in the dorsal and ventral striatal dopamine release. This finding is in line with previous studies which reported locomotor hyperactivity on the 4th and the 10th day of a chronic nicotine exposure [94-95]. The authors of these studies suggested that nicotine-treated rats develop locomotor hyperactivity in response to nicotine, initially due to increases of both the density of dopamine receptors (D1 and D2) and dopamine concentration, and lately due to dopamine receptor supersensitivity in the striatum [102]. The interpretation of the behavioral changes observed following chronic nicotine treatment is somewhat complicated by the observation that the impact of chronic nicotine exposure on locomotion depend upon sex, age, and housing conditions [103-107]. Female animals are less sensitive to the acute and chronic effects of nicotine, but more sensitive to the impact of acute nicotine withdrawal, including the neuroendocrine and behavioral stress responses, when compared to males [103, 105, 108-110]. As regards the locomotor actions of nicotine in male and female Long-Evans and Sprague-Dawley rats, the horizontal activity was more enhanced in Long-Evans females, and the vertical activity was unaltered in Sprague-Dawley males [103]. Also, younger animals exhibit increased sensitivity to the positive, rewarding effects of nicotine and reduced sensitivity to the negative, aversive effects of nicotine withdrawal which may contribute to the higher risk to develop nicotine addiction in adolescents, when compared to adults [73, 107]. As regards the locomotor actions of nicotine, during chronic nicotine administration adolescent males exhibited a greater locomotor activity, when compared to adult males or adolescent females [106]. During nicotine cessation, nicotine-treated adolescent males continued to exhibit greater locomotor activity than saline-treated animals [106]. Another possible factor influencing the results is the housing condition [104]. In saline-treated rats, group housing decreased the horizontal and vertical activity and the center time, a measure of anxiety with effects ocurring sooner in females [104]. For males, nicotine altered both the horizontal and vertical activity, increasing these variables for group-housed males, but decreasing them for individually housed males [104]. For females, nicotine altered only the center time, reducing this measure of anxiety for group-housed females [104]. During nicotine cessation, housing effects appeared more robustly in males and continued in females [104]. Thus, investigation of additional factors, such as sex, age and housing conditions is desirable, but would require more complex experimental design and statistical analysis.

In mice exposed to 7 days of nicotine treatment (after 12 hours following the last nicotine administration) we observed signs of anxiolysis, as mice treated with nicotine spent more time in the open arms of the elevated plus-maze than those treated with saline. This result is supported by a previous study which reported that subchronic administration of nicotine (0.1 mg/kg subcutaneously = SC for 6 days) produces anxiolytic effect in mice [111] and it is opposed by other studies which referred that subchronic (0.3 mg/kg/day nicotine SC for 4 days) or chronic administration (25 mg/kg/day nicotine *via* minipump for 14 days) of nicotine in higher doses than 0.1 mg/kg induces anxiogenic behavior in mice [112-113]. We also observed signs of depression, as mice treated with nicotine spent less time with swimming and climbing in the water than those treated with saline. This result is underlined by previous studies according to which repeated IP nicotine treatment (0.3 mg/kg/day IP for 4 days) produces depression-like behavior [114-115]. Although in our study despite of the significant decrease in the time spent with swimming and climbing, there was no significant difference in the time spent immobile - this being a more typical sign of depression in the forced swim test - in nicotine-treated animals, when compared to the saline-treated animals. Therefore, our results could be rather interpreted as a consequence of the locomotor suppressive effect exerted by nicotine [103-104], than an apparently coexisting anxiolytic and depressive behavior. In addition, the behavioral changes described in mice were not accompanied by significant elevation of the plasma corticosterone concentration.

5.6. The effects of acute nicotine withdrawal

In accordance with the features of the stage of withdrawal/negative affect, in rats exposed to 1 day of nicotine withdrawal (after 24 hours following the last nicotine administration) we expected a decrease of general locomotor activity and a decrease of global striatal dopamine release, which were actually assessed in a previous study following 14 days of nicotine exposure and 24 hours of nicotine withdrawal [100]. Interestingly, in the present experiments only the vertical locomotor activity and the ventral striatal dopamine release were decreased, while the horizontal locomotor activity and the dorsal striatal dopamine release remained increased following acute nicotine withdrawal. The explanation of this finding might be offered by the authors of this previous study, who showed a reduction of the maximum number of D2 receptor sites in the NAcc, but found no alteration of the density and binding affinity of dopamine receptors (D1 and D2) in the putamen and nucleus caudatus [102]. Additionally, the dose and the schedule of the nicotine exposure may also contribute to the difference between the previous and present results. Thus, while tolerance is more likely

to be induced by continuous infusion of nicotine (performed in the previous study), behavioral sensitization is frequently induced by intermittent injection of nicotine (performed in the present study) [96-97]. Nonetheless, continuous exposure to nicotine at doses that result in tolerance to the nicotine-induced sensitization, induces itself a sensitization that is demasked as the tolerance wears off. Hereby tolerance and sensitization must be regarded as two distinct adaptive changes that usually require different conditions, but may also occur following the same dose and schedule of chronic nicotine exposure [96-97]. Consequently, during acute nicotine withdrawal these competing phenomena could be manifested differently between the two subdivisions of the striatum and accordingly, the two aspects of locomotor activity [96-97]. The discrepancies between the behavioral and neurochemical parameters observed following acute nicotine withdrawal is underlined by the differential nicotinic regulation of the nigrostriatal and mesolimbic dopaminergic pathways [116-117]. For example, there are clear differences in the distribution and characteristics of various nAChR subtypes between the dorsal and ventral striatum [116-119]. Acute nicotine exposure increases directly the striatal dopamine neurotransmission *via* presynaptic nAChRs that are α6β2 and/or α4β2 subunit-containing, depending on the brain region [116-117]. The nAChR subtypes that regulate dopaminergic neurotransmission depend critically upon α5 subunits (non-α6 nAChRs) in the dorsal striatum and upon α6 subunits (α6 nAChRs) in the ventral striatum [119]. Chronic nicotine exposure produces no change in the control of dopamine release by α6 relative to non-α6 nAChRs in the putamen and nucleus caudatus, but it induces a downregulation of the α6 nAChRs and an upregulation of non-α6 nAChRs in the NAcc [118]. In addition, nicotine modulates the release of dopamine indirectly, through the release of glutamate and GABA following activation of post-synaptic α7 containing nAChRs [116-119]. Furthermore, there are also differences in the regulation of dopamine release by different nicotinic agonists [116-119]. For instance, nicotine stimulates especially the mesolimbic dopaminergic pathway, in contrast, epibatidine, the most potent nAChR agonist known to date, stimulates preferentially the nigrostriatal pathway [116-117]. The difference in the dose-response curves of the two nicotinic agonists regarding the dorsal and ventral striatal dopamine release suggests different abilities for downregulation and desensitization of the nAChRs found in these brain regions [116-117]. This imbalance in the distribution and the function of nAChRs between the dorsal and ventral striatum might explain the dopamine dysregulation assessed during acute nicotine withdrawal.

Mice exposed to 1 day of nicotine withdrawal (after 24 hours following the last nicotine administration) exhibited signs of anxiety, since the number of entries into the open arms and the time spent in the open arms of the plus-maze decreased in the nicotine-treated group, compared to the saline-treated one. This result is in agreement with previous studies, which showed that acute withdrawal following chronic administration of nicotine (1 day of withdrawal following 0.1 mg/kg/day IP treatment for 14 days or 12-24-48 mg/kg/day treatment *via* minipump for 14 days) precipitates anxiety-like behavior in mice tested in light-dark box or elevated plus-maze. Mice exposed to 1 day of nicotine withdrawal expressed signs of depression as well, since the time spent with swimming and climbing in the water increased in parallel with the time of immobilization in the nicotine-treated group, compared with the saline-treated one. This result coincides with that of a previous study using a similar treatment protocol (2 mg/kg nicotine IP, 4 times/day), following which signs of depression were indicated during acute and chronic nicotine withdrawal in mice investigated in forced swim test [120]. In concordance with these behavioral changes, significant elevation of the plasma corticosterone concentration, reflecting the hyperactivity of the HPA axis, was observed on the 9^{th} day of our study. Indeed, hyperactivity of the HPA axis is associated frequently with nicotine withdrawal syndrome [121-122] and generally with states of anxiety and depression [123-124].

ICV injection of UCN II or UCN III performed in the morning of the 9^{th} day increased the open-arm activity that was previously decreased by acute nicotine withdrawal. Concomitently, ICV injection of UCN II or UCN III reversed the swimming and the climbing activity and the immobility of mice, which were increased and decreased, respectively, by acute nicotine withdrawal. As a matter of fact, the anxiolytic and the antidepressant effects of the urocortins validated in the present study have been already indicated by previous studies using the same methods [35-37]. Additionally, a single administration of UCN II or UCN III attenuated the levels of the plasma corticosterone which were remarkably and significantly augmented on the 9^{th} day in nicotine-treated animals.

6. CONCLUSION

Taken together, this study demonstrate that both selective CRFR1 antagonists, such as antalarmin and selective CRFR2 agonists, such as UCN II and UCN III could be promising candidates as potential therapy in nicotine addiction.

On the one hand, we demonstraded that antalarmin reversed the alterations of the dorsal and ventral striatal dopamine release and the vertical and horizontal locomotor activity which characterize the binge/intoxication phase of nicotine addiction. Actually, these experiments are the first to elucidate that both the rewarding, positive reinforcing effects of chronic nicotine treatment and the aversive, negative effects of acute nicotine withdrawal can be attenuated by administration of selective CRFR1 antagonists, such as antalarmin.

On the other hand, we demonstrated that UCN II and UCN III reduce the anxiety- and depression-like behavior and hyperactivity of the HPA axis that arise during the withdrawal/negative affect phase of nicotine addiction and may persist during the preoccupation/anticipation phase as well. Consequently, these are the first experiments to demonstrate that administration of selective CRFR2 agonists, such as UCN II and UCN III ameliorate the anxiety- and depression-like signs developed following chronic nicotine treatment and the consequent acute nicotine withdrawal, and the hyperactivity of the HPA axis that is associated to them.

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APPENDIX I

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Research report

Changes in striatal dopamine release and locomotor activity following acute withdrawal from chronic nicotine are mediated by CRF1, but not CRF2, receptors

András Buzás, Péter Bokor, Beáta Balangó, Dávid Pintér, Miklós Palotai, Balázs Simon, Krisztina Csabafi, Gyula Telegdy, Gyula Szabó, Zsolt Bagosi*

Department of Pathophysiology, Faculty of Medicine, University of Szeged, Hungary

HIGHLIGHTS

- Male Wistar were exposed to repeated ip injection with nicotine for 7 days.
- On the 8th and the 9th day rats were injected icv with antalarmin or astressin$_{2B}$.
- Horizontal and vertical locomotor activities changed on the 8th and the 9th day.
- Dorsal and ventral striatal dopamine releases changed on the 8th and the 9th day.
- All the changes observed were attenuated by antalarmin, but not astressin$_{2B}$.

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Locomotor activity
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ABSTRACT

The aim of the present study was to investigate the participation of corticotropin-releasing factor (CRF) receptors (CRF1 and CRF2) in the alterations of the dorsal and ventral striatal dopamine release and the vertical and horizontal locomotor activity observed in rats following chronic nicotine treatment and consequent acute withdrawal. In this purpose, male Wistar rats were exposed to repeated intraperitoneal (ip) injection with nicotine or saline solution for 7 days. On the 8th day or the 9th day the rats were injected intracerebroventricularly (icv) with selective CRF1 antagonist antalarmin or selective CRF2 antagonist astressin$_{2B}$ or saline solution. Thirty minutes after the icv injection the changes of the horizontal and vertical locomotor activity were recorded in an *in vivo* conducta system. Immediately after the behavioral recordings the changes of the dorsal and ventral striatal dopamine release were determined in an *in vitro* superfusion system. On the 8th day, the horizontal and vertical locomotor activities and the dorsal and ventral striatal dopamine releases increased significantly in nicotine-treated rats, compared to the saline-treated ones. On the 9th day, the horizontal locomotor activity and the dorsal striatal dopamine release increased significantly, whereas the vertical locomotor activity and the ventral striatal dopamine release decreased significantly in nicotine-treated rats, compared to the saline-treated ones. All the changes observed were attenuated significantly by antalarmin, but not astressin$_{2B}$. The present study demonstrates that the changes of striatal dopamine release and locomotor activity observed following chronic nicotine treatment and consequent acute withdrawal are mediated by CRF1, but not CRF2, receptor.

1. Introduction

Corticotropin-releasing factor (CRF) is a hypothalamic neurohormone, but also an extrahypothalamic neurotransmitter, that regulates the neuroendocrine, autonomic and behavioral stress reponses (Bale et al., 2002; Bale and Vale, 2004; Vale et al., 1981). The actions of CRF are mediated by two distinct G protein-coupled receptors, CRF receptor type 1 (CRF1) and CRF receptor type 2 (CRF2) (Chang et al., 1993; Lovenberg et al., 1995). CRF1 is expressed abundantly in the central nervous system (CNS), including the cerebral cortex, cerebellum and striatum (Van Pett et al., 2000). CRF2 is expressed predominantly in the periphery, and limited centrally to subcortical regions, such as the hypothalamus, hippocampus and amygdala (Van Pett et al., 2000). Originally, it was suggested that CRF1 and CRF2 mediate antagonistic

* Corresponding author at: Department of Pathophysiology, University of Szeged, Semmelweis str. 1, 6725 Szeged, Hungary.
E-mail address: bagosi.zsolt@med.u-szeged.hu (Z. Bagosi).

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effects in the CNS, since stimulation of CRF1 provoked activation of the HPA axis, anxiety and depression, and increase of locomotor activity (at least in a familial environment), whereas stimulation of CRF2 evoked anxiolytic and antidepressant effects, and decrease of locomotor activity (Bale et al., 2002; Bale and Vale, 2004; Vale et al., 1981). Recently, it was demonstrated that the role of CRF receptors in the stress responses is not a matter of simple dualism, but it depends upon the brain regions and neuron populations being activated (Henckens et al., 2016; Janssen and Kozicz, 2013).

Nicotine is the main psychoactive component of tobacco that causes addiction. Besides the regulation of the stress responses, CRF has been implicated in nicotine addiction based on several lines of evidence (Bruijnzeel and Gold, 2005; Sarnyai et al., 2001). First, acute administration of nicotine, like any other stressor, evokes a dose-dependent activation of the hypothalamic–pituitary–adrenal (HPA) axis that is initiated by hypothalamic CRF (Bruijnzeel and Gold, 2005; Sarnyai et al., 2001). Second, nicotine withdrawal syndrome resembles the behavioral stress response that is mediated by extrahypothalamic CRF (Bruijnzeel and Gold, 2005; Sarnyai et al., 2001). Third, exposure to stressors is one of the leading causes of nicotine relapse (Bruijnzeel and Gold, 2005; Sarnyai et al., 2001). Finally, both CRF receptors participate to the acute, chronic and withdrawal actions of nicotine (Bruijnzeel et al., 2009; Bruijnzeel, 2012; George et al., 2007; Kamdi et al., 2009; Marcinkiewcz et al., 2009). The actions of nicotine are mediated by nicotinic acetylcholine receptors (nAchRs) that are considered ligand-gated ion channels composed of pentameric combinations of α and β subunits, since normally they respond to acetycholine and allow natrium or calcium ions to enter the cells (Benowitz, 2010). Based on their primary sites of expression, nAchRs are classified into two subtypes: muscle-type nicotinic receptors found in neuromuscular junctions and neuronal-type nicotinic receptors found on neuronal bodies and nerve terminals (Benowitz, 2010). The most abundant neuronal nAchRs are α4β2, α3β4 and α7 located both pre- and postsynaptically where they can influence the release of other neurotransmitters, such as dopamine, glutamate and GABA (Benowitz, 2010).

Some of the psychoactive actions of nicotine are mediated by the nigrostriatal and mesolimbic pathways (Di Chiara, 2000; Di Chiara et al., 2004). The nigrostriatal pathway originates in the dopaminergic neurons located in the substantia nigra and terminates in the putamen and nucleus caudatus which together constitute the dorsal striatum (Di Chiara, 2000; Di Chiara et al., 2004). The mesolimbic pathway arises from the dopaminergic neurons situated in the ventral tegmental area and projects to the nucleus accumbens that represents the ventral striatum (Di Chiara, 2000; Di Chiara et al., 2004). Classically, the nigrostriatal pathway controls motor behavior, posture and learning of motor programs and habits, whereas the mesolimbic pathway contributes to motor behavior by mediation of reward, emotion and motivation (Everitt and Robbins, 2013). Nevertheless, manipulations of dopamine release in the dorsal and ventral striatum affect motor behavior in distinct, but parallel ways, which depend upon the nature of the cortical and limbic input to these brain structures (Everitt and Robbins, 2013). Acute administration of nicotine increases the release of striatal dopamine that is associated with a sensation of reward and locomotor hyperactivity in rats (Fung and Lau, 1986, 1989). Chronic administration of nicotine also increases the striatal dopamine release, although its impact on the locomotor activity depends upon the dose and schedule of administration (Fung and Lau, 1991, 1992). Usually, continuous infusion of nicotine several times a day may induce tolerance, while repetitive injection once a day may produce sensitization to the effects of nicotine (Di Chiara, 2000; Di Chiara et al., 2004). Nicotine also augments glutamate release, which stimulates the release of dopamine, and GABA release, which inhibits the release of dopamine. With long-term exposure to nicotine, some nAchRs become desensitized, but some do not (Benowitz, 2008, 2010). As a result, GABAergic inhibitory tone diminishes, while glutamatergic excitation continues, thereby increasing excitation of dopaminergic neurons and enhancing responsiveness to nicotine (Benowitz, 2008, 2010). Acute withdrawal following chronic administration of nicotine causes a nicotine withdrawal syndrome that starts promptly within few hours and peaks around 24 h following cessation of chronic nicotine administration (Fung et al., 1996). The nicotine withdrawal syndrome in rats consists of a somatic component, characterized by locomotor hypoactivity, increased appetite and weight gain and an affective component, represented by anxiety, depression and reward deficit (Kenny and Markou, 2001). Some of the affective symptoms, such as anxiety and depression, may persist during chronic nicotine withdrawal (Kenny and Markou, 2001). The basis of nicotine addiction is a combination of positive reinforcement, given by the rewarding, positive effects of nicotine, and negative reinforcement, maintained by the avoidance of the aversive, negative effects of nicotine withdrawal (Benowitz, 2010). The changes of the dorsal and ventral striatal dopamine release can be partly or entirely implicated in both forms of reinforcement, and reflected in the changes of the horizontal and vertical locomotor activity (Di Chiara, 2000; Di Chiara et al., 2004). Therefore, these behavioral and neurochemical parameters can be considered important measures of nicotine addiction (Fung et al., 1996).

The aim of the present study was to investigate the participation of CRF1 and CRF2 in the alterations of the dorsal and ventral striatal dopamine release and the vertical and horizontal locomotor activity observed in rats following chronic nicotine treatment and consequent acute withdrawal. In this purpose, male Wistar rats were exposed to repeated intraperitoneal (ip) injection with 1.4 mg/kg nicotine or saline solution for 7 days, two times/day (at 8:00 and at 20:00). Thus, 12 h passed between the nicotine treatments. This dose and schedule of administration should produce plasma nicotine levels in rats similar to plasma nicotine levels found in an individual who smokes 1–2 packs of cigarettes a day (Benowitz, 2008). In order to assess the behavioral and neurochemical changes induced by chronic nicotine treatment and acute nicotine withdrawal the rats were investigated on the morning of the 8th day (12 h after the last ip administration) and the 9th day (24 h after the last ip administration), respectively. Furthermore, the rats were injected intracerebroventricularly (icv) with selective CRF1 receptor antagonist antalarmin or selective CRF2 receptor antagonist astressin$_{2B}$ or saline solution on the 8th day or the 9th day. Thirty minutes after the icv injection the changes of the horizontal and vertical locomotor activity were recorded in an *in vivo* conducta system. Immediately after the behavioral recordings the changes of the dorsal and ventral striatal dopamine release were determined in an *in vitro* superfusion system.

2. Results

On the 8th day, the horizontal (Fig. 1) and vertical locomotor activity (Fig. 2) and the dorsal (Fig. 3) and ventral striatal dopamine release (Fig. 4) increased significantly in nicotine-treated rats, compared with the saline-treated rats. Tukey post-hoc test revealed the following p values: $p < 0.0001$ for horizontal locomotor activity, $p = 0.0043$ for vertical locomotor activity, $p < 0.0001$ for dorsal striatal dopamine release, and $p < 0.0001$ for ventral striatal dopamine release. All the changes observed on the 8th day were reduced significantly by icv treatment with antalarmin, but not astressin$_{2B}$ (Figs. 1–4). Tukey post-hoc test indicated the following p values: $p = 0.0304$ for horizontal locomotor activity, $p = 0.0002$ for vertical locomotor activity, $p < 0.0001$ for dorsal striatal dopamine release, and $p < 0.0001$ for ventral striatal dopamine release.

On the 9th day, the horizontal locomotor activity (Fig. 1) and the dorsal striatal dopamine release (Fig. 3) were increased significantly, while the vertical locomotor activity (Fig. 2) and the ventral striatal dopamine release (Fig. 4) were decreased significantly in the nicotine-treated rats, compared with the saline-treated rats. Tukey post-hoc test revealed the following p values: $p < 0.0001$ for horizontal locomotor activity, $p = 0.0033$ for vertical locomotor activity, $p < 0.0009$ for

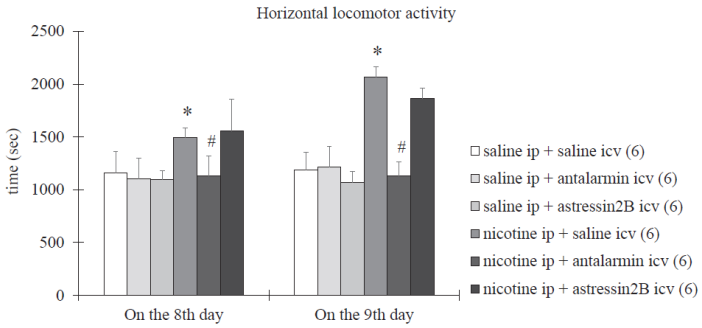

Fig. 1. The horizontal locomotor activity in rats exposed to 7 days of nicotine treatment and 1 day of withdrawal. Behavioral parameters were determined on the 8th and the 9th day. Values are presented as means ± SEM. Statistically significant difference was accepted for $p < 0.05$ and indicated with * for nicotine ip + saline icv vs. saline ip + saline icv and with # for nicotine ip + antalarmin icv vs. nicotine ip + saline icv.

dorsal striatal dopamine release, and $p < 0.0002$ for ventral striatal dopamine release. All the changes assessed on the 9th day were reversed completely by icv treatment with antalarmin, but not astressin$_{2B}$ (Figs. 1–4). Tukey post-hoc test indicated the following p values: $p < 0.0001$ for horizontal locomotor activity, $p = 0.0321$ for vertical locomotor activity, $p = 0.0022$ for dorsal striatal dopamine release, and $p < 0.0001$ for ventral striatal dopamine release. A summary of the effects of the antagonist treatment, the nicotine treatment and the interaction between them is presented in Tables 1–4.

3. Discussion

In rats exposed to 7 days of nicotine treatment (12 h after the last nicotine administration) we observed increases in horizontal and vertical locomotor activity along with increases in the dorsal and ventral striatal dopamine release. This finding is in line with previous studies which reported locomotor hyperactivity on the 4th and the 10th day of a chronic nicotine exposure (Fung and Lau, 1991, 1992). The authors of these studies suggested that nicotine-treated rats develop locomotor hyperactivity in response to nicotine initially due to increases of both the density of dopamine receptors (D1 and D2) and dopamine concentration, and lately due to dopamine receptor supersensitivity in the striatum (Fung and Lau, 1988). The interpretation of the behavioral changes observed following chronic nicotine treatment is somewhat complicated by the observation that the impact of chronic nicotine exposure on locomotion depend upon sex, age, and housing conditions (Faraday et al., 1999b, 2001, 2003a,b, 2005). Female animals are less sensitive to the acute and chronic effects of nicotine, but more sensitive to the impact of acute nicotine withdrawal, including the neuroendocrine and behavioral stress responses, when compared to males (Bangasser and Wiersielis, 2018; Becker, 2016; Faraday et al., 1999a, 2003b, 2005). As regards the locomotor actions of nicotine in male and female Long-Evans and Sprague-Dawley rats, the horizontal activity was more enhanced in Long-Evans females, and the vertical activity was unaltered in Sprague-Dawley males (Faraday et al., 2003b). Also, younger animals exhibit increased sensitivity to the positive, rewarding effects of nicotine and reduced sensitivity to the negative, aversive effects of nicotine withdrawal which may contribute to the higher risk to develop nicotine addiction in adolescents, when compared to adults (Faraday et al., 2003a; Portugal et al., 2012). As regards the locomotor actions of nicotine, during chronic nicotine administration adolescent males exhibited a greater locomotor activity, when compared to adult males or adolescent females (Faraday et al., 2001). During nicotine cessation, nicotine-treated adolescent males continued to exhibit greater locomotor activity than saline-treated animals (Faraday et al., 2001). Another possible factor influencing the results is the housing condition (Faraday et al., 1999b). In saline-treated rats, group housing decreased the horizontal and vertical activity and the center time, a measure of anxiety with effects ocurring sooner in females (Faraday et al., 1999b). For males, nicotine altered both the horizontal and vertical activity, increasing these variables for group-housed males, but decreasing them for individually housed males (Faraday et al., 1999b). For females, nicotine altered only the center time, reducing this measure of anxiety for group-housed females (Faraday et al., 1999b). During nicotine cessation, housing effects appeared more robustly in males and continued in females (Faraday et al., 1999b). Therefore, investigation of additional factors, such as sex, age and housing conditions is desirable, but would require more complex experimental design and statistical analysis.

In rats exposed to 1 day of nicotine withdrawal (24 h after the last nicotine administration) we expected a decrease of general locomotor activity and a decrease of global striatal dopamine release, which were assessed in a previous study following 14 days of nicotine exposure and 24 h of nicotine withdrawal (Fung et al., 1996). Interestingly, in the

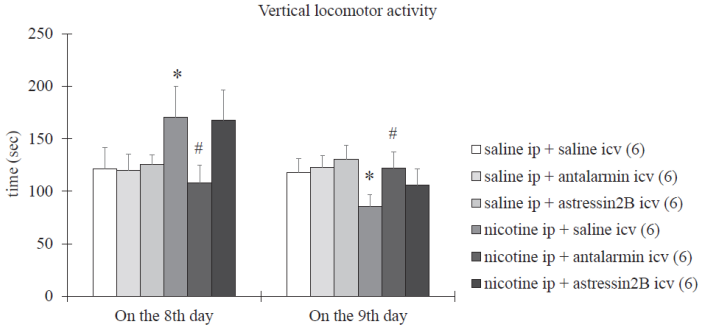

Fig. 2. The vertical locomotor activity in rats exposed to 7 days of nicotine treatment and 1 day of withdrawal. Behavioral parameters were determined on the 8th and the 9th day. Values are presented as means ± SEM. Statistically significant difference was accepted for $p < 0.05$ and indicated with * for nicotine ip + saline icv vs. saline ip + saline icv and with # for nicotine ip + antalarmin icv vs. nicotine ip + saline icv.

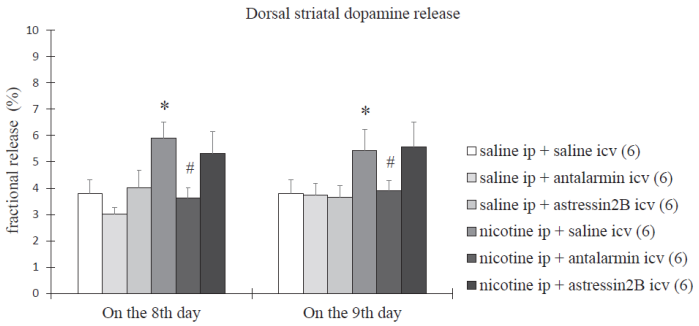

Fig. 3. The dorsal striatal dopamine release in rats exposed to 7 days of nicotine treatment and 1 day of withdrawal. Biochemical parameters were determined on the 8th and the 9th day. Values are presented as means ± SEM. Statistically significant difference was accepted for $p < 0.05$ and indicated with * for nicotine ip + saline icv vs. saline ip + saline icv and with # for nicotine ip + antalarmin icv vs. nicotine ip + saline icv.

present study only the vertical locomotor activity and the ventral striatal dopamine release were decreased, while the horizontal locomotor activity and the dorsal striatal dopamine release remained increased following acute nicotine withdrawal. The explanation of this finding might be offered by the authors of this previous study, who showed a reduction of the maximum number of D2 receptor sites in the nucleus accumbens, but found no alteration of the density and binding affinity of dopamine receptors (D1 and D2) in the putamen and nucleus caudatus (Fung and Lau, 1988). Additionally, the dose and the schedule of the nicotine exposure may also contribute to the difference between the previous and present results. Thus, while tolerance is more likely to be induced by continuous infusion of nicotine (performed in the previous study), sensitization is frequently induced by intermittent injection of nicotine (performed in the present study) (Di Chiara, 2000; Di Chiara et al., 2004). Nonetheless, continuous exposure to nicotine at doses that result in tolerance to the nicotine-induced sensitization, induces itself a sensitization that is demasked as the tolerance wears off. Hereby tolerance and sensitization must be regarded as two distinct adaptive changes that usually require different conditions, but may also occur following the same dose and schedule of chronic nicotine exposure (Di Chiara, 2000; Di Chiara et al., 2004). Consequently, during acute nicotine withdrawal these competing phenomena could be manifested differently between the two subdivisions of the striatum and accordingly, the two aspects of locomotor activity (Di Chiara, 2000; Di Chiara et al., 2004). The discrepancies between the behavioral and neurochemical parameters observed following acute nicotine withdrawal is underlined by the differential nicotinic regulation of the nigrostriatal and mesolimbic dopaminergic pathways (Janhunen et al., 2005; Janhunen and Ahtee, 2007). On one hand, there are clear differences in the distribution and characteristics of various nAchR subtypes between the dorsal and ventral striatum (Exley et al., 2012, 2013; Janhunen et al., 2005; Janhunen and Ahtee, 2007). Acute nicotine exposure increases directly the striatal dopamine neurotransmission *via* presynaptic nAchRs that are α6β2 and/or α4β2 subunit-containing, depending on the brain region (Janhunen et al., 2005; Janhunen and Ahtee, 2007). The nAchR subtypes that regulate dopaminergic neurotransmission depend critically upon α5 subunits (non-α6 nAchRs) in the dorsal striatum and upon α6 subunits (α6 nAchRs) in the ventral striatum (Exley et al., 2012). Chronic nicotine exposure produces no change in the control of dopamine release by α6 relative to non-α6 nAchRs in the putamen and nucleus caudatus, but it induces a downregulation of the α6 nAchRs and an upregulation of non-α6 nAchRs in the nucleus accumbens (Exley et al., 2013). In addition, nicotine modulates the release of dopamine indirectly, through the release of glutamate and GABA following activation of post-synaptic α7 containing nAchRs (Exley et al., 2012, 2013; Janhunen et al., 2005; Janhunen and Ahtee, 2007). On the other hand, there are also differences in the regulation of dopamine release by different nicotinic agonists (Exley et al., 2012, 2013; Janhunen et al., 2005; Janhunen and Ahtee, 2007). For example, nicotine stimulates especially the mesolimbic dopaminergic pathway, in contrast, epibatidine, the most potent nAchR agonist known to date, stimulates preferentially the nigrostriatal pathway (Janhunen et al., 2005; Janhunen and Ahtee, 2007). The differing dose–response curves of the two nicotinic agonists regarding the dorsal and ventral striatal dopamine release suggest different abilities for downregulation and desensitization of the nAchRs found in these brain regions (Janhunen et al., 2005; Janhunen and Ahtee, 2007). This imbalance in the distribution and the function of nAchRs between the dorsal and ventral striatum might explain the dopamine dysregulation assessed during acute nicotine withdrawal.

Taken together, our results demonstrate that the changes of striatal dopamine release and locomotor activity observed following chronic

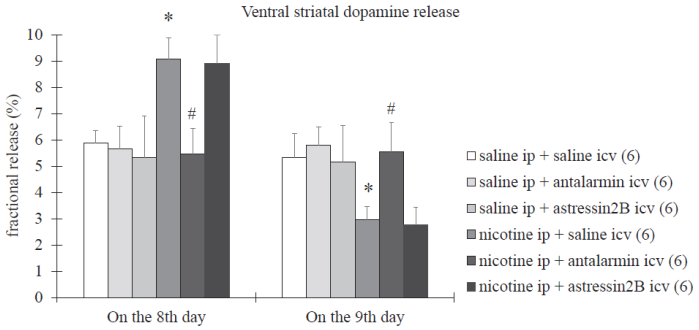

Fig. 4. The ventral striatal dopamine release in rats exposed to 7 days of nicotine treatment and 1 day of withdrawal. Biochemical parameters were determined on the 8th and the 9th day. Values are presented as means ± SEM. Statistically significant difference was accepted for $p < 0.05$ and indicated with * for nicotine ip + saline icv vs. saline ip + saline icv and with # for nicotine ip + antalarmin icv vs. nicotine ip + saline icv.

Table 1
Statistical data for dorsal striatal dopamine release on the 8th and the 9th day.

Source of variation	Degrees of freedom	Sum of squares	Mean square	F value	p value
Dorsal striatal dopamine release on the 8th day					
Antagonist treatment	2.0	16.27	8.133	$F(2, 30) = 24.28$	$p < 0.0001$
Nicotine treatment	1.0	15.61	15.61	$F(1, 30) = 46.58$	$p < 0.0001$
Interaction	2.0	2.950	1.475	$F(2, 30) = 4.40$	$p = 0.0211$
Dorsal striatal dopamine release on the 9th day					
Antagonist treatment	2.0	5.022	2.511	$F(2, 30) = 6.58$	$p = 0.043$
Nicotine treatment	1.0	14.15	14.15	$F(1, 30) = 37.12$	$p < 0.0001$
Interaction	2.0	5.336	2.668	$F(2, 30) = 7.00$	$p = 0.0032$

Table 2
Statistical data for ventral striatal dopamine release on the 8th and the 9th day.

Source of variation	Degrees of freedom	Sum of squares	Mean square	F value	p value
Ventral striatal dopamine release on the 8th day					
Antagonist treatment	2.0	24.91	12.46	$F(2, 30) = 16.77$	$p < 0.0001$
Nicotine treatment	1.0	43.64	43.64	$F(1, 30) = 58.75$	$p < 0.0001$
Interaction	2.0	26.1	13.00	$F(2, 30) = 17.51$	$p < 0.0001$
Ventral striatal dopamine release on the 9th day					
Antagonist treatment	2.0	20.93	10.46	$F(2, 30) = 17.13$	$p < 0.0001$
Nicotine treatment	1.0	25.26	25.26	$F(1, 30) = 41.34$	$p < 0.0001$
Interaction	2.0	9.025	4.513	$F(2, 30) = 7.39$	$p = 0.0025$

Table 3
Statistical data for horizontal locomotor activity on the 8th and the 9th day.

Source of variation	Degrees of freedom	Sum of squares	Mean square	F value	p value
Horizontal locomotor activity on the 8th day					
Antagonist treatment	2.0	351,737	175,869	$F(2, 30) = 4.75$	$p = 0.0161$
Nicotine treatment	1.0	689,453	689,453	$F(1, 30) = 18.62$	$p = 0.0002$
Interaction	2.0	292,703	146,351	$F(2, 30) = 3.95$	$p = 0.0300$
Horizontal locomotor activity on the 9th day					
Antagonist treatment	2.0	1.270e	635,248	$F(2, 30) = 33.49$	$p < 0.0001$
Nicotine treatment	1.0	2.519e	2.519e	$F(1, 30) = 132.81$	$p < 0.0001$
Interaction	2.0	1.671e	835,490	$F(2, 30) = 44.05$	$p < 0.0001$

Table 4
Statistical data for vertical locomotor activity on the 8th and the 9th day.

Source of variation	Degrees of freedom	Sum of squares	Mean square	F value	p value
Vertical locomotor activity on the 8th day					
Antagonist treatment	2.0	8433	4217	$F(2, 30) = 9.31$	$p = 0.0007$
Nicotine treatment	1.0	6241	6241	$F(1, 30) = 13.78$	$p = 0.0008$
Interaction	2.0	6762	3381	$F(2, 30) = 7.47$	$p = 0.0023$
Vertical locomotor activity on the 9th day					
Antagonist treatment	2.0	3627	1814	$F(2, 30) = 10.28$	$p = 0.0004$
Nicotine treatment	1.0	3230	3230	$F(1, 30) = 18.30$	$p = 0.0002$
Interaction	2.0	863.7	431.9	$F(2, 30) = 2.45$	$p = 0.1036$

nicotine treatment and consequent acute withdrawal are mediated by CRF1, but not CRF2. Previous studies have already indicated that blocking CRF1 would prevent some of the affective symptoms (the dysphoria and the reward deficit) (Bruijnzeel et al., 2009, 2012; George et al., 2007; Marcinkiewcz et al., 2009), whereas blocking CRF2 would reverse some of the somatic symptoms (the excessive food intake and the increased body weight) of nicotine withdrawal syndrome in rats (Kamdi et al., 2009). A recent study have also indicated that administration of selective CRF2 agonists could ameliorate the anxiety- and depression-like state developed during acute nicotine withdrawal in mice (Bagosi et al., 2016). The present study completes the previous ones, suggesting that both the rewarding, positive reinforcing effects of nicotine promoted by enhanced striatal dopamine release and the aversive, negative effects of nicotine withdrawal mediated partly by deficient striatal dopamine release could be attenuated by administration of selective CRF1 antagonists. As such, antalarmin may normalize the striatal dopamine release by blocking CRF1 receptors that regulate dopamine neuron firing at the level of the substantia nigra and the ventral tegmental area (Van Pett et al., 2000). Alternatively, antalarmin may inhibit CRF1 receptors located in the dorsal and ventral striatum, but also in the amygdala and hippocampus, from where it can modulate bidirectionally the striatal dopamine release *via* GABAergic and glutamatergic neurotransmission (Bagosi et al., 2006, 2008, 2015; Palotai et al., 2013a,b). The present study does not exclude the possibility that the ability of the selective antagonist of CRF1, but not CRF2, to abolish the behavioral and neurochemical effects of nicotine could simply be due to the differential distribution of CRF1 and CRF2 receptors in the substantia nigra and ventral tegmental area or the dorsal and ventral striatum. Moreover, previous studies demonstrated that CRF1 is expressed abundantly in all these brain regions, whereas CRF2 is limited centrally to the hypothalamus, amydgala, and hippocampus (Van Pett et al., 2000). However, a recent study reported an increased expression of CRF2 in the dorsal striatum after the development of nicotine-induced sensitization in rats (Carboni et al., 2018). In this order of thoughts, both CRF1 and CRF2 must be considered potential targets in the therapy of nicotine addiction.

4. Experimental procedures

4.1. Animals

Male Wistar rats weighing 150–250 g upon arrival were used (N = 80). Although sexually maturized, the rats were considered adolescents, since they were about 6–7 weeks old when the experimental procedures (treatment, *in vivo* procedures, etc.) had started (Sengupta, 2013). Pre-adolescence and adolescence are developmental periods associated with increased vulnerability for nicotine addiction, and exposure to nicotine during these periods may lead to long-lasting changes in behavioral and neuronal plasticity in different brain regions, such as the cerebral cortex, hippocampus and striatum (Philpot et al., 2012; Portugal et al., 2012). In the present study only male animals were used, as previous studies suggested that the behavior of females would be influenced by too many variables, including hormonal fluctuations associated with the female reproductive cycle (Bangasser et al., 2018; Bangasser and Wiersielis, 2018; Becker and Koob, 2016; Dluzen and Anderson, 1997). However, a recent meta-analysis demonstrated that female rats were not more variable regarding behavioral, electrophysiological, neurochemical, and histological measures at any stage of the estrous cycle than male rats (Becker et al., 2016). Thus, future studies should include both male and female rats, and power analyses based on variance in male measures should be sufficient to yield accurate numbers for females as well, even when the estrous cycle is not taken into consideration. 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 nonspecific stress the rats were handled daily. The rats were treated in accordance with the instructions of the Ethical Committee for the Protection of Animals in Research, University of Szeged, Hungary.

4.2. Surgery

The rats were implanted with a stainless steel Luer cannula (10 mm long), aimed at the right lateral cerebral ventricle under anesthesia with 60 mg/kg pentobarbital sodium (Euthanasol, CEVA-Phylaxia, Hungary). 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 the stereotaxic atlas of the rat brain (Pellegrino et al., 1979). Cannulas were secured to the skull with dental cement and acrylate. The rats were allowed for 7 days to recover before experiments were started. After the experiments were concluded, 10 µl of methylene blue (Reanal Ltd., Hungary) at 1 g/100 ml were injected into the lateral cerebral ventricle of the decapitated animals and the position of the cannula was inspected visually. The spread of methylene blue throughout the ventricular space indicated that the whole amount of the CRF antagonist got into the ventricles. Animals without the dye in the lateral cerebral ventricle (8 from 80) were excluded from the final statistical analysis. No histological preparations were performed.

4.3. Treatments

The rats were treated ip with 1.4 mg/kg/10 ml nicotine tartrate (Sigma-Aldrich Inc., USA) or 10 ml/kg of 0.9% saline solution (B. Braun Inc., Germany) for 7 days, two times/day (at 8:00 and at 20:00). One half of the animals were treated icv with 0.1 µg/2 µl antalarmin (Sigma-Aldrich Inc., USA), a selective CRF1 antagonist, or 1 µg/2 µl astressin 2B (Sigma-Aldrich Inc., USA), a selective CRF2 antagonist, or 2 µl of 0.9% saline solution (B. Braun Inc., Germany) on the 8th day (12 h after the last ip administration). The other half of the animals were treated icv on the 9th day (24 h after the last ip administration) based on the same treatment protocol. Hence, rats were divided in 6 groups: group 1 – saline ip + saline icv; group 2 – saline ip + antalarmin icv; group 3 – saline ip + astressin$_{2B}$ icv; group 4 – nicotine ip + saline icv; group 5 – nicotine ip + antalarmin icv; and group 6 – nicotine ip + astressin$_{2B}$ icv.

4.4. In vivo procedure

Thirty minutes after the icv injection, the horizontal and vertical locomotor activities were recorded in an *in vivo* conducta system (MDE, Ltd, Germany), which is based on the principles of the open-field test and was described in our previous studies (Liptak et al., 2013; Szakacs et al., 2015). The apparatus was a square open-field black box with a side length of 60 cm, surrounded by a 40 cm high wall. The floor of the box was divided in 36 (6 × 6) small squares. Five by five rows of photocell beams allowed a computer-based system to register the behavioral activity of each animal. A 60 W light was situated 1 m above the arena floor. Each animal was carried to the experimental room in their home cage and placed in the center of the box with which they were familiarized for 5 min. Then the horizontal activity, representing a measure of overall activity and arousal, and the vertical activity, representing a measure of exploratory and stereotype behavior, were monitorized for 30 min. The box was cleaned between sessions with 96% ethyl-alcohol (Reanal Ltd., Hungary).

4.5. In vitro procedure

After decapitation the changes of dorsal and ventral striatal dopamine releases were determined by an *in vitro* superfusion system (MDE, Ltd, Germany) described in our previous studies (Bagosi et al., 2006; Palotai et al., 2013a). The striatum was isolated and dissected in a Petri dish filled with ice-cold Krebs solution (Reanal, Hungary). The stereotaxic coordinates were 4.0 mm anterior and 1.0 mm posterior to the bregma, according to the stereotaxic atlas of the rat brain (Pellegrino et al., 1979). The dorsal striatum, including the putamen and the nucleus caudatus, and the ventral striatum, including the nucleus accumbens, were decapsulated from the surrounding white matter and separated from each other. Slices of 300 µM were produced with a McIlwain tissue chopper (Campden Instruments Ltd., UK). The slices 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% CO_2 and 95% O_2. During the incubation, the slices were labelled with 15 µmol of [^{3}H]dopamine (GE Healthcare Life Sciences Inc., USA) with a specific activity of 14 Ci/mmol. Two tritiated slices were transferred to each of the four cylindrical perspex chambers of the superfusion system (MDE, Co. Ltd., Germany). Gold electrodes were attached to both halves of the superfusion chambers and connected to an ST-02 electrical stimulator (MDE, Co. Ltd., Germany). A multichannel peristaltic Gilson Minipuls 2 pump (Gilson Inc., USA) was used to maintain a constant superfusion rate of 300 µl/min. The slices were superfused for 30 min to allow tissue equilibrium, and the superfusates were collected in Eppendorf tubes by a multichannel fraction collector Gilson FC 203B (Gilson Inc., USA). After 2 min electrical stimulation consisting of square-wave impulses (voltage: 100 V, pulse length: 5 ms, frequency: 10 Hz) was delivered to each of the four chambers lasting 2 min. The sample collecting lasted 32 min (2 min for each sample) and the peak of the fractional release was observed at 14 min. The remnants of slices were solubilized in 200 ml of Krebs solution, using an ultrasonic homogenizer Branson Sonifier 250 (Labequip Ltd., Canada). The radioactivity in the fractions and the homogenized tissue samples was measured with a liquid scintillation spectrometer (Tri-carb 2100TR, Packard Inc., USA) after the addition of 3 ml of scintillation fluid (Ultima Gold, Packard Inc., USA). The fractional release was calculated as a percentage of the radioactivity present in each collected sample compared to the total radioactivity of the correspondent tissue.

4.6. Statistical analysis

Statistical analysis of the results was performed by analysis of variance (Prism 7 Statistics, GraphPad Inc., USA). The differences between groups were determined by two-way ANOVA followed by Tukey's test for pairwise comparisons when prerequisites were fulfilled. A probability level of 0.05 or less was accepted as indicating a statistically significant difference.

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APPENDIX II

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Brain Research

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Research report

Selective CRF2 receptor agonists ameliorate the anxiety- and depression-like state developed during chronic nicotine treatment and consequent acute withdrawal in mice

Zsolt Bagosi*, Miklós Palotai, Balázs Simon, Péter Bokor, András Buzás, Beáta Balangó, Dávid Pintér, Miklós Jászberényi, Krisztina Csabafi, Gyula Szabó

Department of Pathophysiology, Faculty of Medicine, University of Szeged, Hungary

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ABSTRACT

The aim of the present study was to investigate the effects of the selective agonists of the corticotropin-releasing factor (CRF) 2 receptor, urocortin 2 (UCN 2) and urocortin 3 (UCN 3), on the anxiety- and depression-like signs induced by acute nicotine withdrawal in mice. In order to do so, male CFLP mice were exposed for 7 days to repeated intraperitoneal (IP) injection with nicotine or saline solution and 1 day of acute withdrawal and then a single intracerebroventricular (ICV) injection with UCN 2, UCN 3 or saline solution. After 30 min the mice were observed in an elevated plus-maze test or a forced swim test, for anxiety- and depression-like behavior. After 5 min of testing, the plasma corticosterone concentration reflecting the activity of the hypothalamic-pituitary-adrenal (HPA) axis was also determined by a chemo-fluorescent method. Half of the animals were treated ICV and evaluated on the 8th day, the other half on the 9th day. On the 8th day, nicotine-treated mice presented signs of anxiolysis and depression, but no significant elevation of the plasma corticosterone concentration. On the 9th day, nicotine-treated mice exhibited signs of anxiety and depression and a significant increase of the plasma corticosterone levels. Central administration of UCN 2 or UCN 3 ameliorated the anxiety- and depression-like state including the hyperactivity of the HPA axis, developed during acute withdrawal following chronic nicotine treatment. The present study suggests that selective CRF2 receptor agonists could be used as a therapy in nicotine addiction.

1. Introduction

The urocortins (UCN 1, UCN 2 and UCN 3) are corticotropin-releasing factor (CRF)-related peptides with similar amino acidic structure, but different pharmacological profile. In contrast to CRF that binds preferentially to CRF receptor 1, UCN 1 attaches equipotently to both CRF receptors (Vale et al., 1981; Vaughan et al., 1995), whereas UCN 2 and UCN 3 bind selectively to CRF2 receptor, therefore these are considered selective agonists of the CRF2 receptor (Lewis et al., 2001; Reyes et al., 2001). Central administration of CRF and UCN 1 induces activation of the hypothalamic-pituitary-adrenal (HPA) axis, anxiety-like and depression-like behavior (Bale and Vale, 2004; Vale et al., 1981; Vaughan et al., 1995), while central administration of UCN 2 and UCN 3 produces anxiolytic and antidepressant actions (Tanaka and Telegdy, 2008; Telegdy and Adamik, 2013; Valdez et al., 2002, 2003). Accordingly, activation of the CRF1 receptor, expressed predominantly in the cerebral cortex, the cerebellum and the anterior pituitary, is believed to initiate the endocrine, autonomic and behavioral reactions to stress (Bale and Vale, 2004; Reul and Holsboer, 2002; Van Pett et al., 2000), while activation of the CRF2 receptor, limited centrally to subcortical regions (amygdala, hippocampus, hypothalamus), is thought to terminate these stress responses (Bale and Vale, 2004; Reul and Holsboer, 2002; Van Pett et al., 2000). Actually, the role of CRF2 receptor in the regulation of the HPA axis is still under debate (Fekete and Zorrilla, 2007; Suda et al., 2004), because studies in mice and rats led to contradictory results (Bale et al., 2000, 2002; Jamieson et al., 2006; Maruyama et al., 2007; Pelleymounter et al., 2004).

Besides the regulation of the stress responses, CRF and the urocortins have been implicated in drug addiction (Bruijnzeel and Gold, 2005; Sarnyai et al., 2001). For instance nicotine, the addictive substance of tobacco, can activate the HPA axis, just like any other stressor may do, although its impact on behavior depends on the dose and the time of administration. On the one hand, acute administration

* Correspondence to: Department of Pathophysiology, University of Szeged, Semmelweis str. 1, 6725 Szeged, Hungary.
E-mail address: bagosi.zsolt@med.u-szeged.hu (Z. Bagosi).

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of low doses of nicotine promotes anxiolytic and antidepressant behavior (Andreasen and Redrobe, 2009; Balerio et al., 2005; Varani and Balerio, 2012). On the other hand, acute or chronic administration of higher doses of nicotine provokes anxiety and depression (Bura et al., 2010; Hayase, 2007; Hayase, 2011). In addition, nicotine withdrawal syndrome has an affective component represented by anxiety- and depression-like symptoms (Kenny and Markou, 2001; Markou, 2008; Wonnacott et al., 2005), which are common in the withdrawal phase of all kinds of drug addiction.

Previous studies have already suggested that central administration of UCN 2 or UCN 3 could reverse the alcohol withdrawal-induced anxiety- and depression-like behavior (Valdez et al., 2004; Valdez, 2009). However, up to this date, there is no evidence that selective agonists of CRF2 receptors would ameliorate the affective component of nicotine withdrawal syndrome. Therefore, the aim of the present study was to investigate the effects of UCN 2 and UCN 3 on the anxiety- and depression-like signs induced by chronic nicotine treatment and consequent acute withdrawal in mice. In order to do so, male CFLP mice were exposed for 7 days to repeated intraperitoneal (IP) injection with nicotine or saline solution and 1 day of acute withdrawal and then a single intracerebroventricular (ICV) injection with UCN 2, UCN 3 or saline solution. After 30 min the mice were observed in an elevated plus-maze test or a forced swim test, for anxiety- and depression-like behavior. States of anxiety and depression are usually associated with the hyperactivity of the HPA axis, reflected by increased plasma glucocorticoid concentration (Altemus et al., 1992; Chappell et al., 1996; Nemeroff, 1996a; Plotsky et al., 1998). Consequently, after 5 min of testing, the plasma corticosterone concentration of the mice was also determined by a chemo-fluorescent method. Half of the animals were treated ICV and evaluated on the 8th day, the other half on the 9th day.

2. Results

2.1. Results of the elevated plus-maze test

On the 8th day, the time spent in the open arms/the total time increased significantly, but the number of entries into the open arms/ total number of entries and the total number of entries did not change significantly in nicotine-treated mice compared to the saline-treated ones. The time spent in the open arms/total time increased further after treatment with UCN 2 or UCN 3 in both saline and nicotine-treated animals, but this parameter was increased significantly only in the nicotine-treated group and the rest of the parameters were not influenced significantly in any of the groups (Figs.1-3).

On the 9th day, the number of entries into the open arms/total number of entries and the time spent in the open arms/total time decreased significantly in nicotine-treated mice compared to the saline-treated ones, but the total number of entries was not affected significantly in either the saline-treated or the nicotine-treated group. The decreasing effects were attenuated considerably after treatment with UCN 2 and UCN 3 in the nicotine-treated animals, but not the saline-treated ones and the total number of entries was not affected significantly in either groups (Figs. 1-3).

2.2. Results from the forced swim test

On the 8th day, the swimming and the climbing activity decreased remarkably, but not significantly in nicotine-treated mice, compared to the saline-treated ones, while the time spent immobile was unaltered. After treatment with UCN 2 and UCN 3 the swimming and the climbing activity were further decreased in the nicotine-treated group, while these parameters were increased in the saline-treated group. None of these effects were significant and the last parameter, the time of immobilization was not changed significantly either (Figs. 4-6).

On the 9th day, the swimming and the climbing activity decreased significantly in nicotine-treated mice, compared to the saline-treated ones, and the time of immobilization increased significantly as well. After treatment with UCN 2 or UCN 3 the time spent with climbing and swimming was enhanced and the time spent immobile was reduced in both saline- and nicotine-treated animals, but significant changes were observed only in the nicotine-treated group, and not in the saline-treated group (Figs. 4-6).

2.3. Results from the chemo-fluorescent assay

On the 8th day, the plasma corticosterone concentration was elevated slightly, but insignificantly in the nicotine-treated group, compared to the saline-treated one, and this elevation of the plasma corticosterone level was reversed totally by treatment with UCN 2 and UCN 3. Furthermore, in the saline-treated group the plasma corticosterone levels were reduced by both urocortins (Fig. 7).

On the 9th day, the plasma corticosterone concentration was augmented remarkably and significantly in the nicotine-treated group, compared to the saline-treated one, but this augmentation of the plasma corticosterone level was abolished completely by treatment with urocortins. Nevertheless, in the saline-treated groups the levels of plasma corticosterone were diminished by both UCN 2 and UCN 3 (Fig. 7).

Table 1 shows the results from two-way analysis of variance. The dependent variables were the behavioral parameters or the plasma corticosterone levels and the independent variables were the nicotine treatment and urocortin treatments. Considering nicotine treatment as one factor and urocortin treatment as the other factor, significant interactions between the nicotine and the urocortin treated conditions were observed in the following parameters: the number of the open arms/total number of entries and the time spent with swimming on the 8th and the 9th day, the time spent with climbing on the 8th and the plasma corticosterone concentration on the 9th day.

Table 2 shows the results from multiple analysis of variance. The dependent variables were the behavioral parameters or the plasma corticosterone levels but besides nicotine treatment and urocortin treatments as independent variables, time was also considered. Differences between 12 h *vs* 24 h time slots have been observed in several parameters, such as the number of entries into the open arms, the time spent with swimming, the time spent with climbing, the time spent immobile and the plasma corticosterone concentration.

3. Discussion

Our experiments from the 8th day seem to indicate that acute nicotine withdrawal after 12 h (1/2 day following 2 mg/kg nicotine IP for 7 days, 4 times/day) may evoke anxiolysis, as mice treated with nicotine spent more time in the open arms of the elevated plus-maze

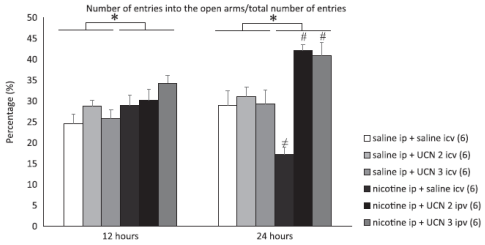

Fig. 1. The effects of UCN 2 and UCN 3 in saline- and nicotine-treated mice investigated for the number of entries into the open arms in an elevated plus-maze test at 12 h and at 24 h of acute nicotine withdrawal following a 7 day-treatment. Values are presented as means ± SEM; statistically significant difference was accepted for $p < .05$ and indicated with ≠ for nicotine IP + saline ICV *vs.* saline IP + saline ICV and with # for nicotine IP + UCN 2 or UCN 3 ICV *vs.* nicotine IP + saline ICV. The interaction between the nicotine and the urocortin condition was significant only on the 9th day and marked with *.

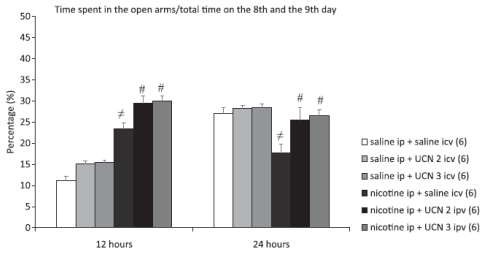

Fig. 2. The effects of UCN 2 and UCN 3 in saline- and nicotine-treated mice investigated for the time spent into the open arms in an elevated plus-maze test at 12 h and at 24 h of acute nicotine withdrawal following a 7 day-treatment. Values are presented as means ± SEM; statistically significant difference was accepted for $p < .05$ and indicated with ≠ for nicotine IP + saline ICV *vs.* saline IP + saline ICV and with # for nicotine IP + UCN 2 or UCN 3 ICV *vs.* nicotine IP + saline ICV. The interaction between the nicotine and the urocortin condition was not significant.

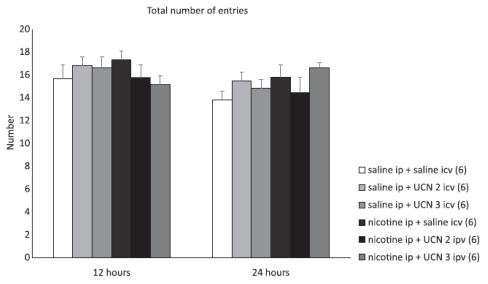

Fig. 3. The effects of UCN 2 and UCN 3 in saline- and nicotine-treated mice investigated for the total time spent into the open and the closed arms in an elevated plus-maze test at 12 h and at 24 h of acute nicotine withdrawal following a 7 day-treatment. Values are presented as means ± SEM; statistically significant difference was accepted for $p < .05$ and indicated with ≠ for nicotine IP + saline ICV *vs.* saline IP + saline ICV and with # for nicotine IP + UCN 2 or UCN 3 ICV *vs.* nicotine IP + saline ICV. The interaction between the nicotine and the urocortin condition was not significant.

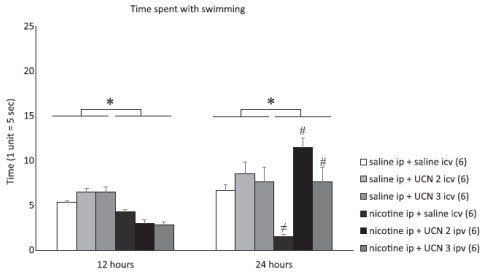

Fig. 4. The effects of UCN 2 and UCN 3 in saline- and nicotine-treated mice investigated for the time spent with swimming in a forced-swimming test at 12 h and at 24 h of acute nicotine withdrawal following a 7 day-treatment. Values are presented as means ± SEM; statistically significant difference was accepted for $p < .05$ and indicated with ≠ for nicotine IP + saline ICV *vs.* saline IP + saline ICV and with # for nicotine IP + UCN 2 or UCN 3 ICV *vs.* nicotine IP + saline ICV. The interaction between the nicotine and the urocortin condition was significant on the 8th and the 9th day and marked with *.

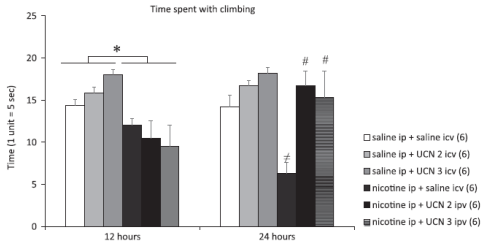

Fig. 5. The effects of UCN 2 and UCN 3 in saline- and nicotine-treated mice investigated for the climbing activity determined in a forced-swimming test at 12 h and at 24 h of acute nicotine withdrawal following a 7 day-treatment. Values are presented as means ± SEM; statistically significant difference was accepted for $p < .05$ and indicated with ≠ for nicotine IP + saline ICV *vs.* saline IP + saline ICV and with # for nicotine IP + UCN 2 or UCN 3 ICV *vs.* nicotine IP + saline ICV. The interaction between the nicotine and the urocortin condition was not significant.

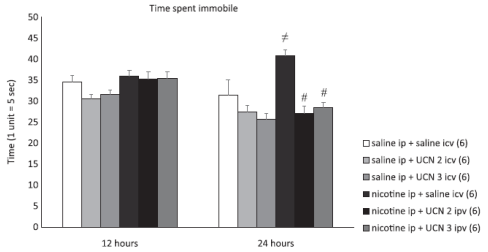

Fig. 6. The effects of UCN 2 and UCN 3 in saline- and nicotine-treated mice investigated for the time spent immobile determined in a forced-swimming test at 12 h and at 24 h of acute nicotine withdrawal following a 7 day-treatment. Values are presented as means ± SEM; statistically significant difference was accepted for $p < .05$ and indicated with ≠ for nicotine IP + saline ICV *vs.* saline IP + saline ICV and with # for nicotine IP + UCN 2 or UCN 3 ICV *vs.* nicotine IP + saline ICV. The interaction between the nicotine and the urocortin condition was not significant.

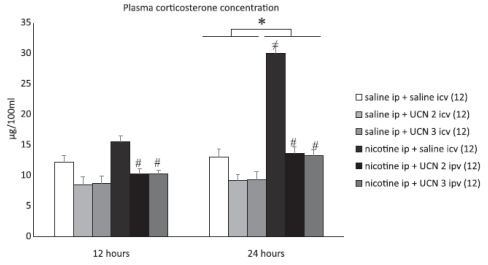

Fig. 7. The effects of UCN 2 and UCN 3 in saline- and nicotine-treated mice investigated for the plasma corticosterone concentration determined with a chemo-fluorescent method at 12 h and at 24 h of acute nicotine withdrawal following a 7 day-treatment. Values are presented as means ± SEM; statistically significant difference was accepted for $p < .05$ and indicated with ≠ for nicotine IP + saline ICV *vs.* saline IP + saline ICV and with # for nicotine IP + UCN 2 or UCN 3 ICV *vs.* nicotine IP + saline ICV. The interaction between the nicotine and the urocortin condition was significant only on the 9th day and marked with *.

than those treated with saline. This result is supported by a previous study that reported that subchronic administration of nicotine (.1 mg/kg subcutaneously, SC, for 6 days) produces anxiolytic effect in mice (Biala et al., 2009) and it is opposed by other studies which referred that subchronic (.3 mg/kg/day nicotine SC for 4 days) or chronic administration (25 mg/kg/day nicotine *via* minipump for 14 days) of nicotine in higher doses than .1 mg/kg induces anxiogenic behavior in mice (Bura et al., 2010; Hayase, 2007). Also, our experiments from the 8th day seem to indicate that acute nicotine withdrawal after 12 h may provoke depression, as mice treated with nicotine spent less time with swimming and climbing in the water than those treated with saline. This result is underlined by previous studies according to which repeated SC nicotine treatment (.3 mg/kg/day IP for 4 days) produces depression-like behavior (Hayase, 2008; Hayase, 2011). Although in

Table 1
Results following two-way analysis of variance (nicotine treatment, urocortin treatment and interaction between them).

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Number of entries into the open arms/total number of entries (12 h)					
Interaction	73,42	2	36,71	F (2, 30) = 1,370	P = 0,2697
UCN	74,42	2	37,21	F (2, 30) = 1,388	P = 0,2651
nicotine	205,6	1	205,6	F (1, 30) = 7,669	P = 0,0095
Residual	804,1	30	26,8		
Number of entries into the open arms/total number of entries (24 h)					
Interaction	1068	2	534	F (2, 30) = 12,62	P = 0,0001
UCN	1299	2	649,6	F (2, 30) = 15,35	P < 0,0001
nicotine	116,4	1	116,4	F (1, 30) = 2,752	P = 0,1076
Residual	1269	30	42,31		
Time spent in the open arms/total time (12 h)					
Interaction	8,858	2	4,429	F (2, 30) = 0,5557	P = 0,5795
UCN	226,9	2	113,5	F (2, 30) = 14,24	P < 0,0001
nicotine	1700	1	1700	F (1, 30) = 213,3	P < 0,0001
Residual	239,1	30	7,97		
Time spent in the open arms/total time (24 h)					
Interaction	101,3	2	50,63	F (2, 30) = 2,746	P = 0,0803
UCN	187,8	2	93,91	F (2, 30) = 5,093	P = 0,0125
nicotine	200,9	1	200,9	F (1, 30) = 10,90	P = 0,0025
Residual	553,1	30	18,44		
Total number of entries (12 h)					
Interaction	17,39	2	8,694	F (2, 30) = 1,586	P = 0,2215
UCN	2,167	2	1,083	F (2, 30) = 0,1976	P = 0,8218
nicotine	0,6944	1	0,6944	F (1, 30) = 0,1266	P = 0,7244
Residual	164,5	30	5,483		
Total number of entries (24 h)					
Interaction	17,06	2	8,528	F (2, 30) = 1,742	P = 0,1924
UCN	5,722	2	2,861	F (2, 30) = 0,5846	P = 0,5636
nicotine	8,028	1	8,028	F (1, 30) = 1,640	P = 0,2101
Residual	146,8	30	4,894		
Time spent with swimming (12 h)					
Interaction	13,39	2	6,694	F (2, 30) = 8,197	P = 0,0014
UCN	0,1667	2	0,08333	F (2, 30) = 0,1020	P = 0,9033
nicotine	66,69	1	66,69	F (1, 30) = 81,67	P < 0,0001
Residual	24,5	30	0,8167		
Time spent with swimming (24 h)					
Interaction	102,4	2	51,19	F (2, 30) = 6,180	P = 0,0057
UCN	213,2	2	106,6	F (2, 30) = 12,87	P < 0,0001
nicotine	4,694	1	4,694	F (1, 30) = 0,5667	P = 0,4574
Residual	248,5	30	8,283		
Time spent with climbing (12 h)					
Interaction	57,06	2	28,53	F (2, 30) = 2,306	P = 0,1171
UCN	2,722	2	1,361	F (2, 30) = 0,1100	P = 0,8962
nicotine	261,4	1	261,4	F (1, 30) = 21,12	P < 0,0001
Residual	371,2	30	12,37		
Time spent with climbing (24 h)					
Interaction	94,39	2	47,19	F (2, 30) = 2,687	P = 0,0845
UCN	333,7	2	166,9	F (2, 30) = 9,499	P = 0,0006
nicotine	113,8	1	113,8	F (1, 30) = 6,477	P = 0,0163
Residual	527	30	17,57		
Time spent immobile (12 h)					
Interaction	16,17	2	8,083	F (2, 30) = 0,6578	P = 0,5253
UCN	36,72	2	18,36	F (2, 30) = 1,494	P = 0,2407
nicotine	100	1	100	F (1, 30) = 8,137	P = 0,0078
Residual	368,7	30	12,29		
Time spent immobile (24 h)					
Interaction	150,1	2	75,03	F (2, 30) = 3,231	P = 0,0536
UCN	648,2	2	324,1	F (2, 30) = 13,96	P < 0,0001
nicotine	136,1	1	136,1	F (1, 30) = 5,861	P = 0,0217
Residual	696,7	30	23,22		
Plasma corticosterone concentration (12 h)					
Interaction	9,527	2	4,763	F (2, 54) = 0,4485	P = 0,6409
UCN	262,7	2	131,3	F (2, 54) = 12,37	P < 0,0001
nicotine	71,11	1	71,11	F (1, 54) = 6,696	P = 0,0124
Residual	573,5	54	10,62		
Plasma corticosterone concentration (24 h)					
Interaction	539,2	2	269,6	F (2, 54) = 21,36	P < 0,0001
UCN	1384	2	692	F (2, 54) = 54,83	P < 0,0001
nicotine	1076	1	1076	F (1, 54) = 85,23	P < 0,0001
Residual	681,5	54	12,62		

(continued on next page)

Table 1 *(continued)*

Abbreviations:
SS = sum of squares.
DF = total degrees of freedom.
MS = mean square.
F (DFn, DFd) = F distribution (degrees of freedom numerator, degrees of freedom denominator).
P value = probability value.

our study despite of the remarkable decrease in the time spent with swimming and climbing, there was no significant difference in the time spent immobile - this being the typical sign of depression in the forced swim test - in nicotine-treated animals, when compared to the saline-treated animals. Therefore, our results could be rather interpreted as a consequence of the locomotor suppressive effect exerted by nicotine (Faraday et al., 1999, 2003), than an apparently coexisting anxiolytic and depressive behavior. In accordance, the behavioral changes described on the 8th day were not accompanied by significant elevation of the plasma corticosterone concentration.

ICV injection of UCN2 or UCN 3 performed in the morning of the 8th day (12 h after the last IP administration) seems to increase further the time spent in the open arms of mice exposed to saline or nicotine treatment. Interestingly, in mice exposed to saline treatment ICV injection of UCN 2 or UCN 3 tends to increase the swimming and the climbing activity, without influencing the time of immobility, in contrast with mice exposed to nicotine treatment, in which it tends to decrease them, without influencing the time of immobility. However, these results should be interpreted in the light of previous experiments, which suggest that the anxiolytic and the locomotor suppressive properties of nicotine are shared by urocortins too (Valdez et al., 2002, 2003). Moreover, a single administration of UCN 2 or UCN 3 lowered the levels of the plasma corticosterone which were slightly elevated on the 8th day in both saline- and nicotine-treated animals, probably due the non-specific stress that is inevitable after testing, despite of the daily handling.

Our experiments from the 9th day demonstrate that acute nicotine withdrawal after 24 h (1 day following 2 mg/kg nicotine IP for 7 days, 4 times/day) produces signs of anxiety, since the number of entries into the open arms and the time spent in the open arms of the plus-maze decreased in the nicotine-treated group, compared to the saline-treated one. This result is in agreement with previous studies, which showed that acute withdrawal following chronic administration of nicotine (1 day of withdrawal following .1 mg/kg/day IP treatment for 14 days or 12–24–48 mg/kg/day treatment *via* minipump for 14 days) precipitates anxiety-like behavior in mice tested in light-dark box or elevated plus-maze. Our experiments from the 9th day also demonstrate that acute nicotine withdrawal after 24 h induces signs of depression, since the time spent with swimming and climbing in the water increased in parallel with the time of immobilization in the nicotine-treated group, compared with the saline-treated one. This result coincides with that of a previous study using a similar treatment protocol (2 mg/kg nicotine IP, 4 times/day), following which signs of depression were indicated during acute and chronic nicotine withdrawal in mice investigated in forced swim test (Mannucci et al., 2006). In concordance with these behavioral changes, significant elevation of the plasma corticosterone concentration, reflecting the hyperactivity of the HPA axis, was

Table 2
Results following multivariate analysis of variance (12 h *vs.* 24 h after the last IP injection).

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
number of entries into the open arms	538,410	2	269,205	F (2, 30) = 7,790	P = 0,001
time spent in the open arms	25,128	2	12.564	F (2, 30) = 0,952	P = 0,392
total number of entries	10,111	2	5,056	F(2, 30)=0,974	P=0,383
time spent with swimming	91,583	2	45,792	F(2, 30)=10,064	P=0.000
time spent with climbing	121,083	2	60,542	F(2, 30)=4,044	P=0,023
time spent immobile	132,194	2	66,097	F(2, 30)=3,723	P=0,030
corticosterone	103,629	2	51,814	F (2, 30) = 4,899	P = 0,011

Pairwise comparisons

Dependent variable: number of entries into the open arms

nicotine	UCN	(I) time	(J) time	Mean Difference (I–J)	Std. Error	Significance	Lower Bound	Upper Bound
saline	saline	12 h	24 h	−4.447	3.394	.195	−11.236	2.342
		24 h	12 h	4.447	3.394	.195	−2.342	11.236
	UCN2	12 h	24 h	−2.223	3.394	.515	−9.012	4.566
		24 h	12 h	2.223	3.394	.515	−4.566	9.012
	UCN3	12 h	24 h	−3.499	3.394	.307	−10.288	3.290
		24 h	12 h	3.499	3.394	.307	−3.290	10.288
nicotine	saline	12 h	24 h	11.751*	3.394	.001	4.962	18.540
		24 h	12 h	−11.751*	3.394	.001	−18.540	−4.962
	UCN2	12 h	24 h	−11.769*	3.394	.001	−18.558	−4.980
		24 h	12 h	11.769*	3.394	.001	4.980	18.558
	UCN3	12 h	24 h	−6.604	3.394	.056	−13.393	.185
		24 h	12 h	6.604	3.394	.056	−.185	13.393

Dependent variable: time spent in the open arms

nicotine	UCN	(I) time	(J) time	Mean Difference (I–J)	Std. Error	Significance	Lower Bound	Upper Bound
saline	saline	12 h	24 h	−16.005*	2.098	.000	−20.202	−11.809
		24 h	12 h	16.005*	2.098	.000	11.809	20.202
	UCN2	12 h	24 h	−13.059*	2.098	.000	−17.256	−8.863
		24 h	12 h	13.059*	2.098	.000	8.863	17.256
	UCN3	12 h	24 h	−12.967*	2.098	.000	−17.164	−8.771
		24 h	12 h	12.967*	2.098	.000	8.771	17.164
nicotine	saline	12 h	24 h	5.781*	2.098	.008	1.584	9.977
		24 h	12 h	−5.781*	2.098	.008	−9.977	−1.584
	UCN2	12 h	24 h	4.102	2.098	.055	−.095	8.298
		24 h	12 h	−4.102	2.098	.055	−8.298	.095
	UCN3	12 h	24 h	3.492	2.098	.101	−.704	7.688
		24 h	12 h	−3.492	2.098	.101	−7.688	.704

Dependent variable: total number of entries

nicotine	UCN	(I) time	(J) time	Mean Difference (I–J)	Std. Error	Significance	Lower Bound	Upper Bound
saline	saline	12 h	24 h	1.833	1.315	.168	−.797	4.464
		24 h	12 h	−1.833	1.315	.168	−4.464	.797
	UCN2	12 h	24 h	1.333	1.315	.315	−1.297	3.964
		24 h	12 h	−1.333	1.315	.315	−3.964	1.297
	UCN3	12 h	24 h	1.833	1.315	.168	−.797	4.464
		24 h	12 h	−1.833	1.315	.168	−4.464	.797
nicotine	saline	12 h	24 h	1.500	1.315	.259	−1.131	4.131
		24 h	12 h	−1.500	1.315	.259	−4.131	1.131
	UCN2	12 h	24 h	1.333	1.315	.315	−1.297	3.964
		24 h	12 h	−1.333	1.315	.315	−3.964	1.297
	UCN3	12 h	24 h	−1.500	1.315	.259	−4.131	1.131
		24 h	12 h	1.500	1.315	.259	−1.131	4.131

Dependent variable: time spent with swimming

nicotine	UCN	(I) time	(J) time	Mean Difference (I–J)	Std. Error	Significance	Lower Bound	Upper Bound
saline	saline	12 h	24 h	−1.333	1.232	.283	−3.797	1.130
		24 h	12 h	1.333	1.232	.283	−1.130	3.797
	UCN2	12 h	24 h	−2.000	1.232	.110	−4.463	.463
		24 h	12 h	2.000	1.232	.110	−.463	4.463
	UCN3	12 h	24 h	−1.167	1.232	.347	−3.630	1.297
		24 h	12 h	1.167	1.232	.347	−1.297	3.630
nicotine	saline	12 h	24 h	2.833*	1.232	.025	.370	5.297
		24 h	12 h	−2.833*	1.232	.025	−5.297	−.370
	UCN2	12 h	24 h	−8.500*	1.232	.000	−10.963	−6.037
		24 h	12 h	8.500*	1.232	.000	6.037	10.963
	UCN3	12 h	24 h	−4.833*	1.232	.000	−7.297	−2.370
		24 h	12 h	4.833*	1.232	.000	2.370	7.297

Dependent variable: time spent with climbing

nicotine	UCN	(I) time	(J) time	Mean Difference (I–J)	Std. Error	Significance	Lower Bound	Upper Bound
saline	saline	12 h	24 h	.167	2.234	.941	−4.302	4.635

(continued on next page)

Table 2 *(continued)*

		24 h	12 h	−.167	2.234	.941	−4.635	4.302
	UCN2	12 h	24 h	−.833	2.234	.710	−5.302	3.635
		24 h	12 h	.833	2.234	.710	−3.635	5.302
	UCN3	12 h	24 h	−.167	2.234	.941	−4.635	4.302
		24 h	12 h	.167	2.234	.941	−4.302	4.635
nicotine	saline	12 h	24 h	5.667*	2.234	.014	1.198	10.135
		24 h	12 h	−5.667*	2.234	.014	−10.135	−1.198
	UCN2	12 h	24 h	−6.167*	2.234	.008	−10.635	−1.698
		24 h	12 h	6.167*	2.234	.008	1.698	10.635
	UCN3	12 h	24 h	−5.833*	2.234	.011	−10.302	−1.365
		24 h	12 h	5.833*	2.234	.011	1.365	10.302
Dependent variable: time spent immobile								
nicotine	UCN	(I) time	(J) time	Mean Difference (I-J)	Std. Error	Significance	Lower Bound	Upper Bound
saline	saline	12 h	24 h	3.000	2.433	.222	−1.866	7.866
		24 h	12 h	−3.000	2.433	.222	−7.866	1.866
	UCN2	12 h	24 h	3.000	2.433	.222	−1.866	7.866
		24 h	12 h	−3.000	2.433	.222	−7.866	1.866
	UCN3	12 h	24 h	6.000*	2.433	.017	1.134	10.866
		24 h	12 h	−6.000*	2.433	.017	−10.866	−1.134
nicotine	saline	12 h	24 h	−4.833	2.433	.052	−9.700	.033
		24 h	12 h	4.833	2.433	.052	−.033	9.700
	UCN2	12 h	24 h	8.167*	2.433	.001	3.300	13.033
		24 h	12 h	−8.167*	2.433	.001	−13.033	−3.300
	UCN3	12 h	24 h	7.000*	2.433	.006	2.134	11.866
		24 h	12 h	−7.000*	2.433	.006	−11.866	−2.134
Dependent variable: corticosterone								
nicotine	UCN	(I) time	(J) time	Mean Difference (I–J)	Std. Error	Significance	Lower Bound	Upper Bound
saline nicotine	saline	12 h	24 h	−1.266	1.878	.503	−5.022	2.489
	UCN2	24 h	12 h	1.266	1.878	.503	−2.489	5.022
	UCN3	12 h	24 h	−.555	1.878	.769	−4.311	3.201
		24 h	12 h	.555	1.878	.769	−3.201	4.311
		12 h	24 h	−.418	1.878	.824	−4.174	3.337
		24 h	12 h	.418	1.878	.824	−3.337	4.174
	saline	12 h	24 h	−16.090*	1.878	.000	−19.845	−12.334
	UCN2	24 h	12 h	16.090*	1.878	.000	12.334	19.845
	UCN3	12 h	24 h	−4.824*	1.878	.013	−8.580	−1.069
		24 h	12 h	4.824*	1.878	.013	1.069	8.580
		12 h	24 h	−5.483*	1.878	.005	−9.238	−1.727
		24 h	12 h	5.483*	1.878	.005	1.727	9.238

Abbreviations: SS = sum of squares.DF = total degrees of freedom.MS = mean square.F (DFn, DFd) = F distribution (degrees of freedom numerator, degrees of freedom denominator). P value = probability value.

observed on the 9th day of our study. Indeed, hyperactivity of the HPA axis is associated frequently with nicotine withdrawal syndrome (Benwell and Balfour, 1979; Rasmussen, 1998) and generally with states of anxiety and depression (Binder and Nemeroff, 2010; Nemeroff, 1996b).

ICV injection of UCN 2 or UCN 3 performed in the morning of the 9th day (at 24 h after the last IP administration) increases the open-arm activity that was previously decreased by acute nicotine withdrawal. Concomitently, ICV injection of UCN 2 or UCN 3 reverses the swimming and the climbing activity and the immobility of mice, which were increased and decreased respectively by acute nicotine withdrawal. As a matter of fact, the anxiolytic and the antidepressant effects of the urocortins validated in the present study have been already suggested by previous studies using the same methods (Tanaka and Telegdy, 2008; Valdez et al., 2002, 2003). Additionally, a single administration of UCN 2 or UCN 3 attenuated the levels of the plasma corticosterone which were remarkably and significantly augmented on the 9th day, at least in the nicotine-treated animals. This attenuation was achieved probably by activation of the CRF2 receptors that are expressed abundantly at the level of the hypothalamus and other subcortical regions (Bale and Vale, 2004; Van Pett et al., 2000).

As regards the differences between the 12 and the 24 h points of acute nicotine withdrawal these are in concert with the appearance of nicotine withdrawal syndrome that usually emerges after 12 h, peaks around 24 h and may even persist for 3 days (Kenny and Markou, 2001). Our results from the 12 h paradigm suggest an altered mood, in form of concomitant signs of anxiolysis and depression, probably due to the non-specific stress that was induced by repeated nicotine treatment that occurred exactly 12 h after the last IP administration from the previous day. Our results from the 24 paradigm, however, suggest that the state of anxiety and depression that is expected during acute nicotine withdrawal is already in full bloom following 1 day. Putatively, the locomotor suppressive effects of nicotine that were exhibited after 12 h of withdrawal may have been masked by the aversive signs expressed after 24 h of withdrawal.

The physiological role of the UCNs in the regulation of the HPA axis is still under debate (Fekete and Zorrilla, 2007; Suda et al., 2004). UCN 1 was proved to increase the plasma ACTH and corticosterone levels in rats (Bagosi et al., 2014; Vaughan et al., 1995), whereas UCN 2 and UCN 3 were shown to induce or not to induce such a stimulatory effect, depending on the species or the strains being administered (Jamieson et al., 2006; Maruyama et al., 2007; Pelleymounter et al., 2004). In contrast, mice deficient for CRF2 receptors displayed signs of anxiety and depression and increased HPA axis activity, suggesting a possible inhibitory action of CRF2 agonists (Bale et al., 2000, 2002; Bale and Vale, 2003). However, our previous experiments suggest that the role of CRF2 receptor in the regulation of the HPA axis can be inhibitory or stimulatory, depending on the actual concentration of their agonists (Bagosi et al., 2013). Thus, the concentration of UCN 2 and UCN 3 used in the present experiments were chosen based on previous experiments in which 2 μl/2 μl of UCN 2 and UCN 3 reduced efficiently the plasma ACTH and corticosterone levels in both Wistar rats and CFLP mice, but the data will be revealed in a future study of ours.

The present study completes previous studies suggesting that

selective CRF2 receptor agonists could be used as a therapy in nicotine addiction. The potential therapeutic role of CRF-related peptides in drug addiction has been proposed earlier. For example, previous studies have already implicated that blocking CRF1 receptors with selective antagonists might prevent the nicotine withdrawal-induced deficit in brain reward function and stress-induced relapse (Bruijnzeel et al., 2007, 2009, 2012; Bruijnzeel, 2012; George et al., 2007). Also, previous studies have insinuated that stimulating CRF2 receptor with selective agonists could reduce the alcohol withdrawal-induced anxiety- or depression-like behavior and alcohol-self administration (Valdez et al., 2004, 2009). Although this is the first study to demonstrate that central administration of UCN 2 and UCN 3 ameliorates the anxiety- and depression-like signs developed during chronic nicotine treatment and consequent acute withdrawal, and the hyperactivity of the HPA axis that is associated to them.

4. Experimental procedures

4.1. Animals

Male CFLP mice weighing 24–30 g were used. During the experiments they were kept and handled in accordance with the instructions of the University of Szeged Ethical Comittee for the Protection of Animals in Research. The animals were housed in their home cages at constant room temperature (23 °C) on a standard illumination schedule, with 12-h light and 12-h dark periods (lights on from 6:00 a.m.). Commercial food and tap water were available *ad libitum*. The mice were allowed for 7 days to acclimatize before surgery and they were handled daily to minimize the effects of non-specific stress.

4.2. Surgery

The mice were implanted with a polyethylene Luer cannula (6 mm long) aimed at the right lateral cerebral ventricle under anesthesia with 60 mg/kg of sodium pentobarbital (Euthasol, CEVA-Phylaxia, Hungary). The stereotaxic coordinates were .5 mm posterior and .5 mm lateral to the bregma, and 3 mm deep from the dural surface. Cannulas were secured to the skull with cyanoacrylate containing instant glue, they were closed by a metal string between injections. The mice were allowed for 5 days to recover after the surgery. After the end of the experiments, 2 µl of methylene blue was injected *via* the cannula of decapitated animals to check the permeability and the right position. Data from animals with improper cannula were excluded from statistical analysis.

4.3. Treatments

The mice (N=72) were treated IP with 2 mg/kg/10 ml nicotine or 10 ml/kg saline solution for control for 7 days, 4 times/day. Half of the mice were treated ICV on the 8th day (12 h after the last IP treatment), the other half on the 9th day (24 h after the last IP treatment) with 2 µg/2 µl UCN 2, 2 µg/2 µl UCN 3 or 2 µl of saline solution for control. Thus, mice were divided in 6 groups based on the following treatments: 1. saline IP + saline ICV, 2. saline IP + UCN 2 ICV, 3. saline IP + UCN 3 ICV, 4. nicotine IP + saline ICV, 5. nicotine IP + UCN 2 ICV and 6. nicotine IP + UCN 3 ICV. Nicotine solution was obtained from nicotine hydrogen tartrate salt (Sigma-Aldrich Inc, USA), while doses of UCN 2 and UCN 3 were diluted from trifluoroacetate salts of UCN 2 and UCN 3, respectively (Bachem Ltd., Switzerland) with saline solution of .9% NaCl (B. Braun Inc., Germany).

4.4. Elevated plus-maze test

Thirty minutes after the ICV administration, half of the mice (N=36) were evaluated for anxiety-like behavior in an elevated plus-maze test for 5 min. Half of these mice were tested on the 8th day and the other half on the 9th day. The wooden apparatus, described previously by Pellow et al. (1985), consists of a plus-shaped platform elevated at 638 mm from the floor, made-up by four opposing arms of 87 mm×155 mm. Two of the opposing arms are enclosed by 163 mm-high side and end walls (closed arms), whereas the other two arms have no walls (open arms). Each mouse was placed in the central area (10 cm×10 cm) of the maze, facing one of the open arms and entry into an arm was defined as the entry of all four feet of the animal into that arm. 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. For 5 min period the following parameters were recorded by an observer sitting at 1 m distance from the center of the plus-maze: 1. the ratio between the number of entries into the open arms and the total number of entries, 2. the ratio between the time spent in the open arms and the total time and 3. the total number of entries.

4.5. Forced swim test

Thirty minutes after the ICV administration, half of the mice (N=36) were evaluated for depression-like behavior in a forced swim test for 5 min. Half of these mice were tested on the 8th day and the other half on the 9th day. The apparatus, described originally by Porsolt et al. (1977), consists of a plexiglass cylinder of 200 mm height and 120 mm diameter, containing 1.5 l of water. Each mouse was dropped individually into the 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. In parallel, their attempt to escape the cylinder by climbing or swimming may decrease or cease eventually. For 5 min period the following parameters were recorded by an observer sitting at 1 m distance from the center of the plus-maze: 1. the climbing activity (the time that mice spent with climbing the walls, in their attempt to escape the cylinder), 2. the swimming activity (the time that mice spent with swimming in the water, in their attempt to remain at the surface) and 3. the time of immobilization (the time that mice spent in an upright position on the surface with its front paws together). A 5 s period was considered a time unit, therefore the activity and the immobility were expressed in time units.

4.6. Chemofluorescent assay

After 5 min of testing, all the mice were decapitated and their trunk blood was collected for determination of the plasma corticosterone concentration. Actually, half of the mice were sacrificed on the 8th day, the other half on the 9th day. The method used for this determination is a chemo-fluorescent assay that was described by Zenker and Bernstein (1958) and modified by Purves and Sirett (1965). The trunk blood was collected into heparinized tubes and centrifuged for 10 min at 3000 rpm for determination of the plasma corticosterone levels. 200 ml aliquots of the medium were transferred to centrifuge tubes. A reagent blank of 200 µl of distilled water and 2 corticosterone standards of the same volume containing 25 µg or 50 µg, respectively were prepared. 5 ml of methylene chloride was delivered with an automatic pipette to each tube and rocked for 30 min to allow complete extraction of corticosterone by the solvent. The extract is 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. 4 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 min, centrifuged at 3000 rpm. For 10 min and was allowed to stand at room temperature for 2 h, which permitted the maximum development of fluorescence from

corticosterone. Emission intensity was measured from the lower sulfuric acid layer with Hitachi 204-A fluorescent spectrophotometer at 456 nm extinction and 515 emission wave-length. The concentration of corticosterone of the samples was calculated from the values of the standards and expressed in μg/100 ml.

4.7. Statistical analysis

Statistical analysis of the results was performed by analysis of variance (GraphPad Prism, GraphPad Software Inc., USA). The differences between groups were determined by two-way ANOVA or MANOVA followed by a Tukey post-hoc test and a probability level of .05 or less was accepted as indicating a statistically significant difference.

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