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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

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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

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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

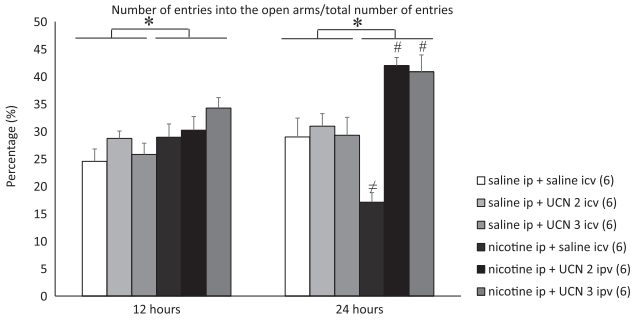

Fig. 1. The effects of UCN 2 and UCN 3 in saline- and nicotine-treated mice investigated for the number of entries into the open arms in an elevated plus-maze test at 12 h and at 24 h of acute nicotine withdrawal following a 7 day-treatment. Values are presented as means ± SEM; statistically significant 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 *.

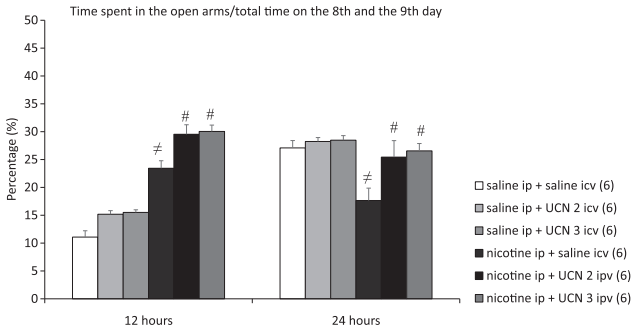

Fig. 2. The effects of UCN 2 and UCN 3 in saline- and nicotine-treated mice investigated for the time spent into the open arms in an elevated plus-maze test at 12 h and at 24 h of acute nicotine withdrawal following a 7 day-treatment. Values are presented as means ± SEM; statistically significant difference 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.

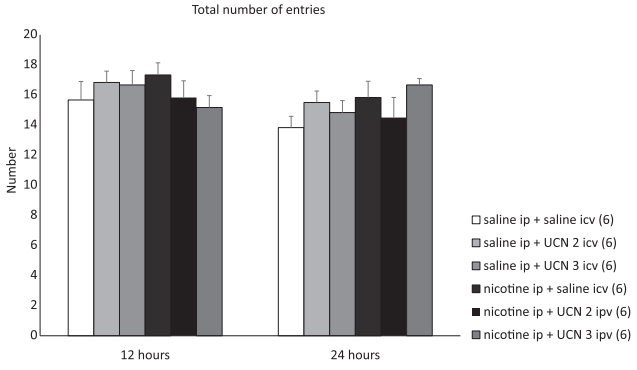

Fig. 3. The effects of UCN 2 and UCN 3 in saline- and nicotine-treated mice investigated for the total time spent into the open and the closed arms in an elevated plus-maze test at 12 h and at 24 h of acute nicotine withdrawal following a 7 day-treatment. Values are presented as means ± SEM; statistically 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.

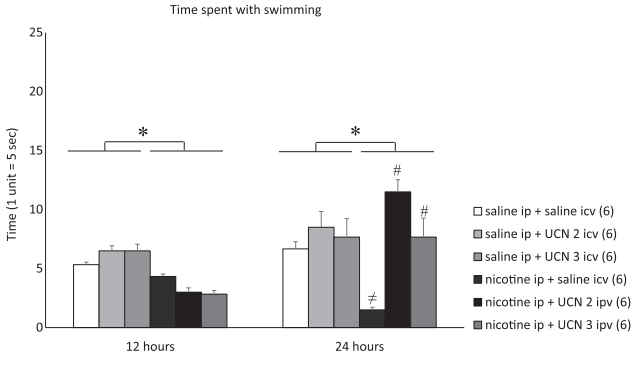

Fig. 4. The effects of UCN 2 and UCN 3 in saline- and nicotine-treated mice investigated for the time spent with swimming in a forced-swimming test at 12 h and at 24 h of acute nicotine withdrawal following a 7 day-treatment. Values are presented as means ± SEM; statistically significant difference was 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 *.

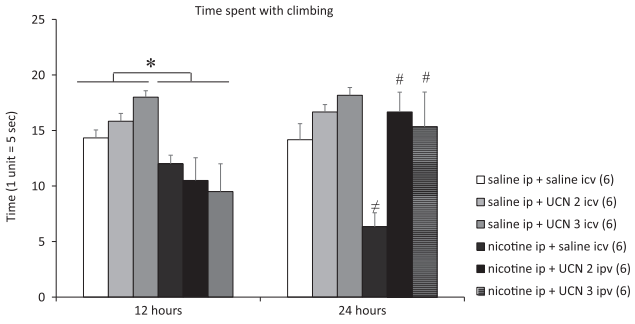

Fig. 5. The effects of UCN 2 and UCN 3 in saline- and nicotine-treated mice investigated for the climbing activity determined in a forced-swimming test at 12 h and at 24 h of acute nicotine withdrawal following a 7 day-treatment. Values are presented as means ± SEM; statistically significant difference 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.

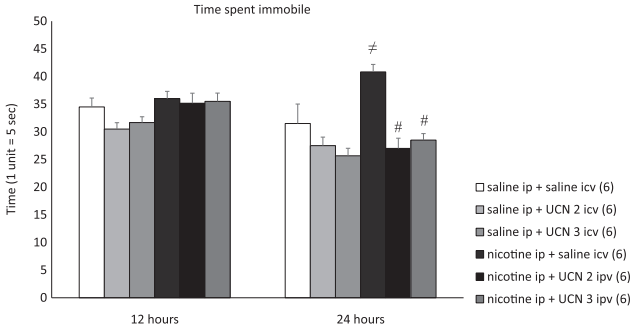

Fig. 6. The effects of UCN 2 and UCN 3 in saline- and nicotine-treated mice investigated for the time spent immobile determined in a forced-swimming test at 12 h and at 24 h of acute nicotine withdrawal following a 7 day-treatment. Values are presented as means ± SEM; statistically significant difference 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.

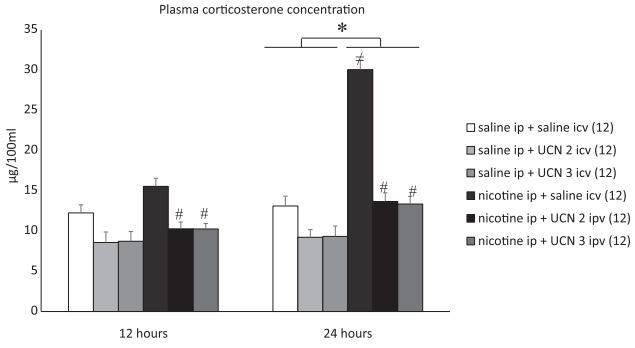

Fig. 7. The effects of UCN 2 and UCN 3 in saline- and nicotine-treated mice investigated for the plasma corticosterone concentration determined with a chemo-fluorescent method at 12 h and at 24 h of acute nicotine withdrawal following a 7 day-treatment. Values are presented as means ± SEM; statistically 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		

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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