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Enkephalin analog, cyclo[N^{ε},N^{β}-carbonyl-D-Lys^2,Dap^5] enkephalinamide (cUENK6), inhibits the ethanol withdrawal-induced anxiety-like behavior in rats

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ABSTRACT

An analog of enkephalin, cyclo[N^{ε},N^{β}-carbonyl-D-Lys^2,Dap^5] enkephalinamide (cUENK6), is predominantly a functional agonist of μ-opioid receptors (MOPr) and, to a lesser extent, of δ-opioid receptors (DOPr) *in vitro*. The aim of the present study was to determine whether cUENK6 could affect ethanol withdrawal-induced anxiety-like behavior in the elevated plus maze (EPM) test in rats. An anxiety-like effect of withdrawal was predicted to occur in the EPM test 24 h after the last ethanol administration (2 g/kg, intraperitoneally [i.p.]; 15% w/v once daily for 9 days). Ethanol withdrawal decreased the percent of time spent by rats in the open arms and the percent of open-arms entries. cUENK6 (0.25 nmol), given by intracerebroventricular (i.c.v.) injection, significantly reversed these anxiety-like effects of ethanol withdrawal and elevated the percent of time spent by rats in the open arms and the percent of open-arms entries. These effects of cUENK6 were significantly inhibited by the DOPr antagonist naltrindole (NTI) (5 nmol, i.c.v.), but not by the MOPr antagonist β-funaltrexamine (β-FNA) (5 nmol, i.c.v.). The preferential DOPr agonist [Leu^5]-enkephalin (LeuEnk) (2.7 and 5.4 nmol, i.c.v.) and the MOPr agonist morphine (6.5 and 13 nmol, i.c.v.) reduced the anxiety-like effects of ethanol withdrawal. cUENK6 at the dose of 0.25 nmol did not disturb locomotor activity in the EPM, in contrast to cUENK6 at the dose of 0.5 nmol, and morphine at 6.5 and 13 nmol. However, similarly to LeuEnk, cUENK6 induced the anxiolytic-like effects in naïve rats. Thus, our study suggests that cUENK6 reduced ethanol withdrawal-induced anxiety-like behavior by activation of δ-opioid receptors rather than μ-opioid receptors.

Introduction

Excessive ethanol consumption over a prolonged period of time results in the development of ethanol dependence that is a chronic relapsing brain disorder, characterized by the loss of control of ethanol consumption (Heilig, Egli, Crabbe, & Becker, 2010). Abrupt discontinuation of ethanol intake leads to the abstinence syndrome, characterized by increased anxiety and physical symptoms, such as tremors, nausea, sweating, elevated heart rate, and convulsions (Heilig et al., 2010). Drug withdrawal anxiety-like behavior may be due to activation of the structures involved in the modulation of anxiety, such as the amygdala, the hypothalamus, and the dorsolateral periaqueductal gray matter (Graeff, Silveira, Nogueira, Audi, & Oliveira, 1993). However, the nature of the relationship between ethanol withdrawal and anxiety is still not well understood. Ethanol withdrawal-induced anxiety-like behavior is thought to be the most important negative motivation that leads to relapse to alcohol use (Schulteis & Liu, 2006; Willinger et al., 2002).

Ethanol acts on many cellular targets, involving several neuromodulators within many neural networks in the brain (Krystal & Tabakoff, 2002). Despite this complex activity, the role of the endogenous opioid system in the effects of ethanol seems to be significant. The opioid system consists of four opioid receptors (the μ [MOPr], δ [DOPr], κ [KOPr], and receptors for nociceptin/orphaninFQ [NOPr]) and four precursor peptide genes, which encode endorphins, enkephalins, dynorphins, and nociceptin/orphaninFQ (Akil et al., 1984; Cox et al., 2000). Ethanol can modulate the activity of the endogenous opioid system by altering the release, synthesis, and post-translational processing of endogenous opioid peptides, and by influencing the interactions of peptides with their specific receptors (Dave, Eiden, Karanian, & Eskay, 1986). The involvement of opioid receptors and opioid peptides in ethanol consumption, reward, and dependence has been documented (Herz, 1997). On the

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other hand, a blockade of the opioid receptors can reduce the rewarding effect and, in consequence, the consumption of ethanol, both in animals and humans (Krystal et al., 2001). Therefore, the opioid antagonists, such as nalmefene or naltrexone, are effective for treatment of alcoholism (Mason, Salvato, Williams, Ritvo, & Cutler, 1999).

Published data indicate the essential role of δ-opioid receptors and endogenous ligands of these receptors (enkephalins in the physiological control of emotion- and motivation-related behaviors) (Nieto, Guen, Kieffer, Roques, & Noble, 2005). Moreover, opioid peptides are also involved in mediating some of the rewarding effects of ethanol (Gianoulakis, 2004; Roberts et al., 2001) and the expression of anxiety-like behavior after cessation of ethanol administration (Esel et al., 2001; Kiefer, Horntrich, Jahn, & Wiedemann, 2002). Some authors suggest that anxiety, depression, and convulsions, observed during withdrawal of ethanol, are caused by decreased levels of endogenous enkephalins and β-endorphin (Aguirre, Del Arbol, Raya, Ruiz-Requena, & Rico Irles, 1990; Esel et al., 2001; Kiefer et al., 2002; Vescovi, Coiro, Volpi, Giannini, & Passeri, 1992).

Cyclo[Nε,Nβ-carbonyl-d-Lys2, Dap5] enkephalinamide (cUENK6) is a new synthetic enkephalin analog. In cUENK6, the ring formation was achieved via a ureido group, incorporating the ω amino group of d-Lys2 and the β amino group of Dap5 in d-Lys2, Dap5-enkephalinamide. This enkephalin analog contains an 18-membered ring structure, and is one of the most potent functional MOPr agonists among the enkephalin analogs reported, as determined in the guinea pig ileum assay (IC50 = 0.212 nM) (Pawlak et al., 2001). In comparison with LeuEnk, this analog was functionally about 1200 times more potent as an agonist toward MOPr. Furthermore, in the mouse vas deferens assay (IC50 = 0.651), the peptide was about 17 times more potent as a functional agonist toward DOPr than LeuEnk (Pawlak et al., 2001). Thus, these results imply that cUENK6 is predominantly a functional agonist of MOPr and, to a lesser extent, of DOPr *in vitro*.

Taking into account the above data, which suggest an involvement of the opioid system in ethanol effects, the aim of the present study was to investigate the influence of cUENK6, the new enkephalin analog, on the ethanol withdrawal-induced anxiety-like behavior in rats. Furthermore, LeuEnk and morphine were used as preferential DOPr and MOPr agonists, respectively, to determine the effect of these compounds on the ethanol withdrawal-induced anxiety-like behavior.

Materials and methods

Animals

Male Wistar rats (HZL, Warsaw, Poland, 200–250 g) were used in all experiments. The animals were housed under standard laboratory conditions (22 °C) with a 12 h light/dark cycle (lights on from 7:00 A.M. to 7:00 P.M.) in groups of four rats per cage. The animals were allowed a 7-day period for acclimation before onset of the experiment, with access to standard food (Bacutil, Motycz, Poland) and water *ad libitum*. The experiments were performed between 8:00 A.M. and 4:00 P.M. All procedures were performed in agreement with ethical regulations and were approved by the local European Ethics Committee.

Anxiety-like effect of ethanol withdrawal

The method was based on the procedure described by Cole, Littleton, and Little (2000) in mice but has been modified for rats and described in our previous paper (Kotlinska & Bochenski, 2008; Kotlinska, Pachuta, Bochenski, & Silberring, 2009). At the beginning of the experiment, the rats were randomly divided into two experimental groups. For 9 consecutive days, saline was given intraperitoneally (i.p.) to one group (control) of rats, and ethanol, at the dose of 2 g/kg (15% w/v, i.p.), was administered to the other groups, once daily, at 8.00 A.M. In our study, the rats were additionally made ethanol-dependent by enforced consumption of 4% (w/v) ethanol solution in drinking water (available at home cages instead of water) during the experiment. All animals had free access to standard food during the development of ethanol dependence. Each day the rats were weighed and observed, and the amount of consumed ethanol solution was recorded. The intake of ethanol was approximately 30 mL of 4% (w/v) solution per rat during 24 h (~1.2 g/kg). During the experiment, the rats in the ethanol group lost about 20% of their body weight. On the last day of ethanol administration, the ethanol solution was removed and the rats were injected i.p. with a dose of 2 g/kg ethanol to standardize the time of ethanol withdrawal. The control animals received an equivalent volume of saline. The rats were provided with free access to water and food after removal of the 4% (w/v) ethanol solution. On the 10th day of the experiment, 24 h after the last ethanol injection, the animals were tested in the elevated plus maze (EPM) for 5 min during ethanol withdrawal. The estimation of time of onset of the anxiety-like behavior was based on earlier published results, in which recorded maximum anxiety-like effects occurred in rats after the first 24 h of abstinence (Bhattacharya, Chakrabarti, Sandler, & Glover, 1995; Pandey, Zhang, Mittal, & Nayyar, 1999).

Apparatus

The test was performed according to the method described by Pellow and File (1986). The plus-shaped maze was made of wood and positioned at a height of 50 cm above the floor. Two opposite arms were open (50 × 10 cm) and the other two were enclosed with walls (50 × 10 × 40 cm). The experiments were carried out in a darkened and quiet room with a constant light of 15 W, located 80 cm above the maze and directed toward the apparatus. The light levels on the open and enclosed arms were equal. Three days before the experiment, each rat was handled every day for 5 min. The plus-maze experiment was initiated by placing a rat at the center of the plus maze, facing the open arm, after which the number of entries and the time spent in each of the two arms were recorded for a period of 5 min. The "arms entry" was recorded when a rat entered the arm with all four paws. The maze was carefully cleaned with tap water after each test session.

The anxiety-like effect of ethanol withdrawal was measured for each rat as a) the time spent in the open arms as a percent of the total time spent exploring both the open and closed arms (Percent Time) and b) the number of entries into the open arms as a percentage of the total number of entries into both open and closed arms (Percent Entries). Furthermore, the locomotor activity of rats was evaluated as c) the total number of entries into the closed arms of the plus-maze apparatus. The results were scored by an experimenter blind to the conditions of the study.

Drugs

Absolute ethanol (Polmos, Poznan, Poland) was mixed with 0.9% NaCl (saline) to make a 15% w/v solution, which was injected i.p. or mixed with drinking water to make a 4% (w/v) solution.

Cyclo[Nε,Nβ-carbonyl-d-Lys2, Dap5] enkephalinamide (cUENK6), the first *enkephalin analog* cyclized *via a ureido group,* was synthesized as described earlier (Pawlak et al., 2001). The crude product was purified to homogeneity by semi-preparative reverse-phase high-performance liquid chromatography (RP-HPLC) on a Vydac C-18 column (250 × 10 mm), using the following system: solvent