ETHANOL WITHDRAWAL IN RATS IS ATTENUATED BY INTRACEREBROVENTRICULAR ADMINISTRATION OF NEUROPEPTIDE Y

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Abstract — Aims: The effects of intracerebroventricular administration of neuropeptide Y (NPY) on ethanol withdrawal were studied in rats. Methods: Ethanol was administered intragastrically five times daily for 4 days. At 16–17 h after the last infusion of ethanol, rats were rated for withdrawal using a score based on three signs: irritability, tremor and rigidity. Subsequently, the rats received an injection of NPY (12 or 24 nmol) or vehicle and were rated for signs of withdrawal. Results: At both doses, NPY significantly reduced ethanol withdrawal, the effect of the larger dose being more pronounced. Conclusions: Our results are consistent with the concept that NPY receptors are centrally involved in the regulation of neuronal excitability and might form a novel therapeutic target for treatment of ethanol withdrawal and other states of neuronal hyperexcitability.

INTRODUCTION

Neuropeptide Y, a 36-residue polypeptide widely distributed in the central nervous system, has been implicated in a wide range of biological functions, including feeding, anxiety, epilepsy, depression, circadian rhythms, memory, and cardiovascular regulation (Heilig and Widerlöv, 1990; Gehlert, 1998). There is increasing evidence that neuropeptide Y is also involved in neuronal mechanisms of ethanol misuse. For instance, NPY-deficient mice drink more ethanol than wild-type mice (Thiele et al., 1998, 2000). Conversely, transgenic mice overexpressing NPY have lower preference for ethanol (Thiele et al., 1998). Rats genetically selected for alcohol preference display lower levels of NPY-like immunoreactivity in the amygdala, hippocampus and frontal cortex, as compared to non-prefering rats (Ehlers et al., 1999a; Hwang et al., 1999). Intracerebroventricular (i.c.v.) administration of NPY produces electrophysiological effects similar to those of ethanol in rats (Ehlers et al., 1998b, 1999) and decreases ethanol intake in alcohol-prefering rats (Badia-Elder et al., 2001). In humans, a gene variant with substitution of proline (7) with leucine (7) in the signal peptide part of NPY has been associated with increased ethanol intake (Kauhanen et al., 2001). Recently, a polymorphism of the NPY gene located at the 5671 locus of exon 3 was associated with human alcohol withdrawal seizures (Okubo and Harada, 2001).

NPY receptors are potential pharmacological targets for treatment of several neuropsychiatric disorders. For instance, it has been shown in our laboratory (Woldbye et al., 1996, 1997; Woldbye, 1998; Klemp and Woldbye, 2001) and by others (Vezzani et al., 1999; Reibet al., 2001) that exogenous application of NPY inhibits epileptic and epileptiform seizures as well as opioid withdrawal (Woldbye et al., 1998; Clausen et al., 2001). Both seizures (Meldrum, 1994) and opioid withdrawal (Aghajanian et al., 1994) are associated with increased release of glutamate and neuronal hyperexcitability. Ethanol withdrawal is a serious medical condition also characterized by neuronal hyperexcitability and increased glutamate release (Tsai and Coyle, 1998). Gamma-aminobutyric acid A receptor-modulating drugs, such as benzodiazepines and barbiturates, are efficient at reducing ethanol withdrawal symptoms in humans and rodents (Kram and Rafaelsen, 1978; Ulrichsen et al., 1986, 1995). However, these drugs may not yield sufficient protection against the neurotoxic effects of withdrawal, and glutamate antagonists have therefore been suggested as a potential alternative treatment strategy (Tsai and Coyle, 1998). NPY inhibits presynaptically the release of glutamate in hippocampal slices of rats (Vezzani et al., 1999) and humans (Patrylo et al., 1999). Thus NPY receptors might be a potential target for alternative treatment of withdrawal. The purpose of the present study was therefore to test whether exogenous application of NPY can attenuate symptoms of ethanol withdrawal in rats.

MATERIALS AND METHODS

Male Wistar rats (Mol.WIST Han, Møllegården, DK; 280–315 g), kept under standard laboratory conditions, were used. All experimental procedures were performed in accordance with the Danish Regulation for Animal Research. Rats were anaesthetized with an i.p. injection of equithesin (3.3 ml/kg; SAD, Denmark) and a cannula for i.c.v. injection was stereotaxically implanted into the right lateral ventricle (coordinates: 2.0 mm from bregma on the coronal suture of the skull, 4.5 mm below the surface of the skull) (Woldbye et al., 1997). Three or four days later, all rats received intragastric infusions of ethanol (200 g/l) five times daily between 08:00 and 24:00 for 4 days (Majchrowicz, 1975; Ulrichsen et al., 1986). The dose of ethanol was adjusted individually on each intubation session to keep the intoxication score at 3–5 according to the severity scale of Majchrowicz (1975); 0 = neutrality; no signs of intoxication; 1 = sedation; reduced muscle tone, dulled appearance, and slow locomotor activity, but no impairment of gait or coordination; 2 = ataxia 1: slight gait impairment and slight motor incoordination but able to elevate abdomen and pelvis; 3 = ataxia 2: clearly impaired staggering gait and impaired coordination, some elevation of abdomen and pelvis; 4 = ataxia 3: slowed righting reflex, heavily impaired

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coordination, no elevation of abdomen and pelvis; 5 = loss of righting reflex: unable to right itself when placed on its back, other reflexes still present; 6 = coma: no signs of movement, no response to pain stimuli, no blinking reflex, spontaneous breathing. Initially, all rats received 6 g/kg ethanol. During the rest of the intoxication period the ethanol dose was decided as follows based on intoxication scores: sedation: 4.5–5.5 g/kg; ataxia 1: 3.5–4.5 g/kg; ataxia 2: 2.5–3.5 g/kg; ataxia 3: 1–2.5 g/kg; loss of righting reflex: 0–1 g/kg; coma: 0 g/kg. Once the animals had received the first dose of ethanol, the intoxication score was ≥1 for the rest of the intoxication period. Ethanol fed to the rats was dissolved in a solution of Ringer also containing sucrose (300 g/l) and a multivitamin mixture (4 ml/l). Sucrose was added to avoid hypoglycaemia and ketosis (Hemmingsen and Chapman, 1980).

At 16–17 h after the last infusion of ethanol, all rats were scored for signs of withdrawal according to the following rating scale (Ulrichsen et al., 1986). Three signs were noted: irritability, rigidity, and tremor. Each sign was given a score of 0–3 (0 = not present; 1 = slight; 2 = moderate; 3 = severe). Withdrawal rating was repeated 15 min later to establish baseline levels for each animal. Rats with a mean total withdrawal score of ≤4 were excluded at this point. Assessed on the mean baseline score, the rats were divided into two groups using stratified randomization (Altman, 1991) to ensure equal inter-group withdrawal levels. One group received an i.c.v. injection (10 µl) containing human/rat synthetic NPY (12 or 24 nmol; #N-5017, Sigma, St Louis, MO, USA) while the other group received vehicle (0.9% isotonic saline and 1% bovine serum albumin) administered for a duration of 1 min. The two different doses of NPY were tested in separate experiments. Fifteen minutes later and subsequently every 15 min, the withdrawal score was determined during the next 2 h and again at 5 h after the i.c.v. injection. Subsequently, the rats were killed. Withdrawal rating was done by an observer blinded as to whether NPY or vehicle had been injected. Rats developing seizures during the observation period were excluded because the withdrawal score, post-ictally, was not believed to represent truly the effects of withdrawal per se, considering the possible influence of post-ictal behavioural depression.

For each rat, the withdrawal score was plotted as a function of time. Visual inspection of these plots made it reasonable to assume a linear relationship between the withdrawal score and the time from 15 min to 2 h post-i.c.v. For each rat, the ‘withdrawal slope’ of the line that would best fit the data was calculated by linear regression analysis (Altman, 1991). The ‘withdrawal slopes’ and the withdrawal score at 5 h post-i.c.v. were statistically analysed using Mann–Whitney U-tests with \( P < 0.05 \) as the level of significance.

RESULTS

There were no differences in the total ethanol dose and mean intoxication scores between the NPY groups and corresponding vehicle groups: 12 nmol NPY [mean ethanol dose (± SD) = 36.9 ± 4.4 g/kg and mean intoxication score = 3.0 ± 0.3] and corresponding vehicle group: 37.4 ± 6.1 g/kg and 3.0 ± 0.3; 24 nmol NPY (38.3 ± 4.8 g/kg and 3.3 ± 0.2), and corresponding vehicle group: 37.1 ± 4.2 g/kg and 3.4 ± 0.2).

The total withdrawal scores during the observation period are shown in Fig. 1. For both NPY doses, the withdrawal score of NPY-treated rats was lower than vehicle from 75 min post-i.c.v., reaching its lowest level at ~105 min. The ‘withdrawal slopes’ are shown in Fig. 2. The slopes of the total withdrawal score of both 12 and 24 nmol NPY were significantly steeper (\( P < 0.006 \)) than those of the vehicle groups, indicating an anti-withdrawal effect of NPY at both doses. The withdrawal score following 24 nmol was significantly lower at 5 h post-i.c.v. whereas the 12 nmol dose was not (Fig. 1).

As to the effects on individual withdrawal signs, a significant reduction was found for both NPY doses on irritability, but significance was only reached at 24 nmol on tremor (Fig. 2). The slopes of the rigidity score were not significantly decreased by either dose of NPY, but a trend was noted at 24 nmol (\( P = 0.074 \)). A total of three out of 16 rats receiving NPY, as opposed to two out of 17 receiving vehicle, developed seizures during the 5 h observation period. This would appear to rule out an

Fig. 1. Total ethanol withdrawal scores calculated by rating the items irritability, rigidity, and tremor. Each item was assigned a score of 0–3. Values are medians with 25th and 75th percentiles as error bars. Effects of neuropeptide Y (NPY) at 5 h post-i.c.v. injection were analysed separately using the Mann–Whitney U-test. Other time points of NPY application were analysed using linear regression (see Fig. 2). \( #P < 0.02 \) vs vehicle.
inhibitory effect of NPY on withdrawal seizures. However, this matter cannot be determined in the present study, because withdrawal seizures in the NPY groups occurred before anti-withdrawal effects of NPY were most pronounced. In addition, the number of convulsing rats in the vehicle groups was too small, in the first place, to allow the detection of an anticonvulsant effect.

**DISCUSSION**

The present study shows that i.c.v. administration of NPY at doses of 12 and 24 nmol/rat attenuates symptoms of ethanol withdrawal in rats. The effect appeared to be more pronounced and of a longer duration at the higher dose, suggesting the effect to be dose dependent. The reason why the anti-withdrawal effect of NPY did not become evident until ~75 min post-i.c.v. is unclear. The anti-seizure effect of NPY against hippocampal electrographic seizures is present already at 20 min post-i.c.v. (Woldbye et al., 1996; Klemp and Woldbye, 2001). Likewise, the anti-withdrawal effect of NPY on morphine withdrawal is present at 30 min post-i.c.v. (Woldbye et al., 1998). Possibly, more widespread diffusion of NPY is required in the present model for NPY to exert its effect.

The elucidation of the receptors mediating the anti-withdrawal effects of NPY in the present study awaits the testing of specific NPY receptor ligands. Y1, Y2 and Y5, which are the quantitatively most important NPY receptors in the rat brain, are all potential candidates. Thus, inhibitory effects of NPY against morphine withdrawal are mediated by receptors with a Y5-like receptor profile (Woldbye et al., 1998). Both Y5 and Y2 receptors have been implicated in reduction of neuronal hyperactivity and hyperexcitability associated with seizures (Woldbye et al., 1997; Vezzani et al., 1999). The Y1 receptor is another candidate, because it appears to mediate anti-anxiety effects of centrally administered NPY (Heilig et al., 1989).

Several studies indicate that increased activity at glutamatergic synapses may be centrally involved in the cerebral mechanisms of the ethanol withdrawal syndrome. Thus, in rodents chronically treated with ethanol, glutamatergic N-methyl-D-aspartate (NMDA) receptors are up-regulated in the hippocampus (Trevisan et al., 1994) and glutamate-induced neurotoxicity is increased in cerebellar granule cells (Iorio et al., 1993). Moreover, increased sensitivity to the neurotoxic and convulsant action of NMDA is present during ethanol withdrawal (Sanna et al., 1993), and mice bred selectively for their ability to display ethanol withdrawal seizures have increased density of NMDA receptor binding sites (Valverius et al., 1990). In hippocampal slices, NPY inhibits neuronal activity by presynaptically reducing the release of glutamate (Vezzani et al., 1999). As the release of glutamate during ethanol withdrawal is increased in several forebrain regions, including the hippocampus (Harms and Woodward, 1995; Rosetti and Carboni, 1995; Daehlou and De Witte, 1999), reduction of glutamate release seems to be one likely mechanism for the anti-withdrawal effect of NPY in the present study.

Because benzodiazepines and barbiturates presently used in the clinic to treat ethanol withdrawal may not sufficiently antagonize the neurotoxic effects of withdrawal (Tsai and Coyle, 1998), drugs with antagonistic effects at glutamate receptors have been suggested as an alternative treatment. In this context, the present study indicates that NPY receptors might deserve attention as a future target for treatment of ethanol withdrawal.

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![Fig. 2. Scatterplots showing the effects of doses of 12 and 24 nmol of neuropeptide Y (NPY)/rat on ethanol withdrawal scores in two separate experiments. Values are individual slopes calculated for each rat by linear regression from withdrawal scores during a period of 15 min to 2 h post-i.c.v. injection. **P < 0.01 vs vehicle, Mann–Whitney U-test. Veh., vehicle; horizontal short dark lines, means; ns, not significant.](image-url)
REFERENCES


