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Neutral endopeptidase and alcohol consumption, experiments in neutral endopeptidase-deficient mice

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Abstract

Alcohol consumption was investigated in mice which were rendered deficient in the peptide-degrading enzyme neutral endopeptidase (EC 3.4.24.11) (NEP −/−) by gene targeting and compared to alcohol consumption in corresponding wild type mice (NEP +/+). Mice were offered a free choice to drink tap water or 10% alcohol. The NEP −/− mice consumed significantly more alcohol (≈42%) than the NEP +/+ mice, whereas no significant differences were observed in the total fluid consumption. The daily food consumption of alcohol naive NEP −/− animals was elevated (≈29%). Furthermore, the activities of peptidases closely related to neutral endopeptidase were analysed ex vivo in several brain regions from NEP −/− and NEP +/+ mice not treated with alcohol. There was no obvious compensation for the total loss of neutral endopeptidase by the functionally related peptidases angiotensin-converting enzyme and aminopeptidase N. In vitro, the degradation of exogenously applied [Leu^5]enkephalin was not reduced in membrane preparations of those brain regions assayed in NEP −/− mice. A small reduction in [Leu^5]enkephalin degradation was detected in striatal membrane preparations of NEP −/− mice, if aminopeptidase N was additionally blocked by bestatin or amastatin.

Keywords: Neutral endopeptidase; Enkephalinase; Enkephalin; Knockout mouse; Alcohol consumption; Angiotensin-converting enzyme; Enkephalin-degrading peptidases; Opioid

1. Introduction

A better understanding of the molecular mechanisms involved in the motivation to drink alcohol and in the pathogenesis of alcohol-related diseases is considered an important prerequisite for the development of new and more effective therapies. When comparing alcohol with other narcotics, the development of new therapeutic strategies seems to be more difficult because no receptors for alcohol are known. Peptidergic systems (opioids, angiotensins, kinins, tachykinins, neuropeptide Y) are known to play a crucial role in the motivation to drink alcohol as well as in the development and maintenance of alcohol disease. Therefore, it was suggested that peptide-degrading enzymes, which are co-localized with neuropeptide receptors in the brain (Waksman et al., 1986), are also involved in alcohol disease-related processes. Whereas a great number of neuroreceptors is known to date, only a few peptidases are involved in neuropeptide degradation (Turner, 1990; Turner and Tanzawa, 1997; Konkoy and Davis, 1996). Among them, angiotensin-converting enzyme (EC 3.4.15.1), aminopeptidase N (EC 3.4.11.2), and neutral endopeptidase (EC 3.4.24.11) play an essential role. Whereas the relationship of angiotensin-converting enzyme (Grupp et al., 1991; Grupp, 1992; Grupp and Chow,

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1992; Fitts, 1993) and aminopeptidase N (Szczepanska and Grupp, 1993; Szczepanska et al., 1996a,b) to alcohol consumption and addiction have been described in detail in the recent years, that of neutral endopeptidase has still been somewhat neglected.

As reviewed by Turner and Tanzawa (1997), neutral endopeptidase is a widely distributed, but not ubiquitous enzyme. It is expressed in many tissues (brain, kidney, testis, respiratory tract, adrenal cortex) and cells (lymphocytes, granulocytes, spermatozoa, endothelial cells). Neutral endopeptidase acts as an endopeptidase, cleaving peptide bonds at the amino side of hydrophobic amino acid residues. Enkephalins, kinins (e.g. bradykinin), tachykinins (e.g. substance P), atrial natriuretic peptide, interleukin-1, and endothelins are typical substrates. Neutral endopeptidase is a type II membrane protein, i.e. it consists of a short N-terminal cytoplasmic domain (27 amino acids) and a long extracellular C-terminal domain (more than 700 amino acids). The C-terminal domain contains a cysteine-rich cluster (12 residues) near the cellular membrane, and the ''zincin motif'' (HExxH), responsible for the coordinate binding of a Zn^{2+} ion near the C-terminus. The highly conserved neutral endopeptidase gene (> 80 kb) is localized on chromosome 3 (q21–q27) in the human genome and contains 24 exons. It produces three different mRNA species, which differ only in their non-coding regions. In contrast to angiotensin-converting enzyme, isoforms of neutral endopeptidase are not known (Schulz et al., 1991; Turner and Tanzawa, 1997).

Among the rare reports insinuating a relationship between alcohol and neutral endopeptidase, the following papers are important. George et al. (1991) used the genetically determined high preference for alcohol consumption of C57BL/6J mice as a model for alcohol disease and compared the enkephalin degradation in this mouse strain with that of DBA/2 mice, which strongly avoided alcohol. Enkephalin was degraded more quickly in striatal regions of the alcohol-preferring C57BL/6 mice. I.c.v. application of kelatorphan, a mixed-inhibitor of neutral endopeptidase and aminopeptidase N, significantly reduced enkephalin degradation as well as alcohol consumption. Panchenko et al. (1984) and Beliaev et al. (1984) characterized the ''enkephalinase A'' activity in rats after alcohol consumption. Blum et al. (1988) proposed to reduce alcohol (as well as polydrug) consumption by application of an ''enkephalinase inhibiting cocktail — SAAVE'' consisting of D-phenylalanine and vitamins. Finally, recent results of Frette et al. (1998) indicate that chronic alcohol consumption is associated with elevated neutral endopeptidase activity in human serum.

Neutral endopeptidase gene knockout mice (NEP −/−) (Lu et al., 1995) have prompted new opportunities for further investigation of the relationship between alcohol and neutral endopeptidase. The homozygous neutral endopeptidase deficient animals appear quite normal, apart from a greater sensitivity to endotoxic shock and an elevated microvascular permeability (Lu et al., 1995, 1997). Saria et al. (1997) measured the enkephalin levels in brain regions and different tissues of these animals and surprisingly, found no elevations, but significant reductions compared to NEP +/+ mice. The reduction in enkephalins may be caused by decreased biosynthesis resulting from increased negative feedback regulation in the absence of enkephalin degradation by neutral endopeptidase. On the other hand, the enkephalin outflow by K^+-challenge is significantly elevated in striatal slices of NEP −/− mice (Saria et al., 1999). It is not known whether the total absence of neutral endopeptidase will result in altered levels of other neutral endopeptidase degradable endogenous neuropeptides.

The present study was initiated to characterize the influence of the total elimination of neutral endopeptidase activity on drinking behaviour and alcohol consumption. Furthermore, we were interested to find out whether the knockout of the neutral endopeptidase gene results in compensation by functionally related peptidases in different brain regions and how these peptidases respond to alcohol consumption. Finally, we were interested to know whether the complete loss of neutral endopeptidase results in reduced enkephalin degradation.

2. Methods

A total of 60 male animals (30 NEP −/− mice (Lu et al., 1995) and 30 corresponding NEP +/+ mice) were maintained under barrier conditions and housed at a 12 h light–dark cycle at 20°C in plastic boxes with two animals each. At the beginning, the animals were 10 weeks old and had a mean weight of 26.6 ± 0.4 g (NEP +/+) and 24.9 ± 0.4 g (NEP −/−). The animals had no experience with alcohol at the beginning of the drinking experiment. The daily alcohol and water consumption, as well as the changes in the body weight, were monitored over 4 weeks. Two groups of mice (15 NEP −/− and 15 NEP +/+ mice) had free choice to drink out of two bottles, which contained either 10% (v/v) alcohol or tap water. Two additional groups of mice were treated comparably to the mice offered ethanol, except they were only offered tap water in both of the two bottles. Following exposure to ethanol and/or tap water, mice were decapitated. Membrane preparations (Hulme, 1992) were obtained from selected brain regions (Popov et al., 1973) from all four groups of animals. The protein content of the membrane preparations was measured using the method of Bradford (1976). In another experiment, the daily food intake was measured over three weeks in alcohol naive animals (12 NEP −/− and 12 NEP +/+ mice).

Measurements of angiotensin-converting enzyme, aminopeptidase N, and neutral endopeptidase activities were carried out as described earlier: aminopeptidase N

was measured fluorimetrically using Tyr-AMC as substrate (Heder et al., 1992) the specificity of the reaction was characterized by amino-peptidase suppression with 10^{-4} M bestatin (Sigma). Angiotensin-converting enzyme was measured fluorimetrically using Hip-His-Leu as substrate (Siems et al., 1985). The specificity of the reaction was characterized by angiotensin-converting enzyme suppression with 10^{-6} M lisinopril (Merck, Sharp and Dohme). Neutral endopeptidase activity was measured by high performance liquid chromatography (HPLC) monitoring of [D-Ala^2,Leu^5]enkephalin degradation and formation of Tyr-D-Ala-Gly in the presence of 10^{-4} M bestatin and 10^{-6} M lisinopril (Winkler et al., 1998); the specificity of the reaction was characterized by neutral endopeptidase suppression with 10^{-6} M phosphoramidon (Sigma).

[Leu^5]enkephalin degradation by membrane preparations was investigated by incubation of 100 μM [Leu^5]enkephalin in 50 mM Tris-buffer (pH 7.4) with a final protein concentration of 0.5 mg/ml. The reaction was stopped by addition of 0.35 M $HClO_4$. The remaining [Leu^5]enkephalin concentration was quantified by HPLC (Shimadzu, isocratic elution with acetonitril [25%] and 75% $NaClO_4$ [0.15 M]/NaH_2PO_4 [0.01 M] buffer [pH 2.2], nucleosil column [100 C18]) at 216 nm.

3. Results

The drinking experiment did not reveal significant differences between NEP +/+ mice and NEP −/− mice in their total fluid consumption. In our experiments, the NEP +/+ mice drank 300.1 ± 5.7 g fluid/kg body weight/day (mean ± S.E.M.), and the NEP −/− mice drank 297.3 ± 7.4 g fluid/kg body weight/day. The daily water consumption of both groups of untreated animals (water in two bottles) was also not different. We measured 262.2 ± 3.9 g water/kg body weight/day (mean ± S.E.M.) for NEP +/+ and 275.2 ± 7.9 g water/kg body weight/day for NEP −/− mice, respectively. Furthermore, there were no different tendencies in the development of body weight between the both groups of mice (data not shown).

The daily food intake was measured in alcohol naive mice. The NEP −/− mice consumed significantly more (194.2 ± 12.1 g/kg body weight) in comparison with the NEP +/+ mice (149.8 ± 12.5 g/kg, $P < 0.05$).

The mean alcohol consumption expressed in g 10% alcohol/kg/day is presented in Fig. 1A. The crucial value for the specific inclination of the animals to imbibe alcohol is the relative proportion of alcohol intake to total fluid intake, as shown in Fig. 1B. Both values were significantly ($P < 0.01$) higher in NEP −/− mice than in NEP +/+ mice. The development of ethanol intake for NEP +/+ and NEP −/− over the last 3 weeks is shown in Fig. 1C.

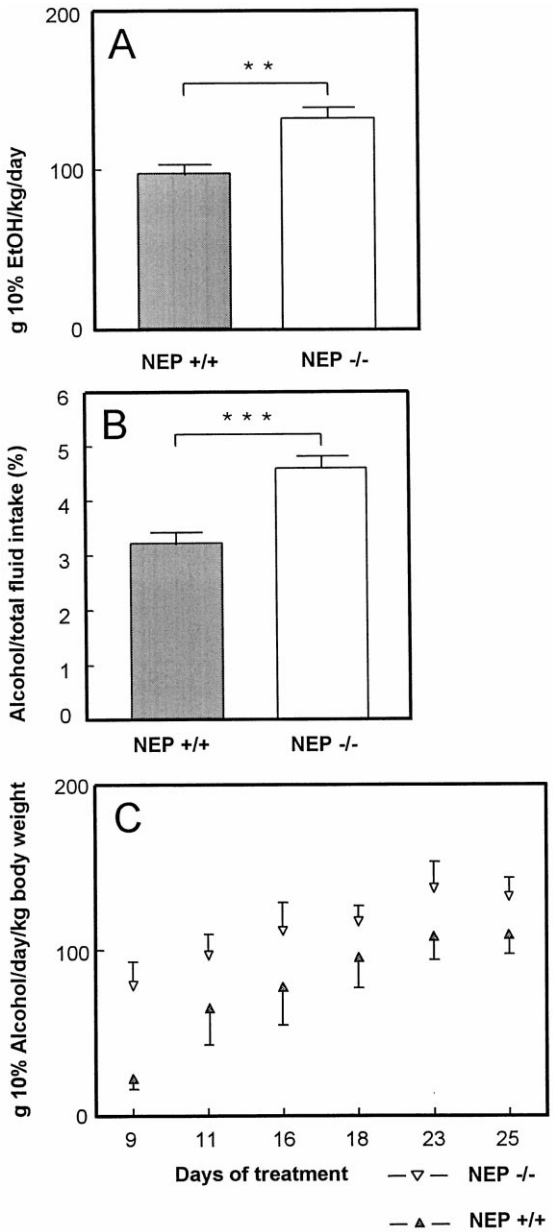

Fig. 1. Voluntary alcohol consumption in 15 NEP −/− and 15 NEP +/+ mice. The drinking experiment was performed after stabilization of drinking as a free choice test with tap water and 10% unsweetened alcohol in tap water (mean of the last 10 24-h drinking sessions, ± S.E.M.; $^{**}P < 0.01$, $^{***}P < 0.001$, calculated by two-sided *t*-test). (A) Alcohol consumption per kg body weight and day. (B) Percentage of pure alcohol consumption related to the total fluid intake. (C) Development of ethanol intake for NEP +/+ and NEP −/− over the last three weeks of the experiment (mean values of all animals; S.E.M.). The difference of the both curves ($P < 0.01$) was confirmed by two-way ANOVA.

The analysis of variance (two-way ANOVA) indicates that both curves are different ($P < 0.01$, $F(1,92) = 11.04$), and the amount of consumed alcohol increases during the observation period ($P < 0.01$, $F(5,92) = 5.25$).

Minor differences in peptidase activities were noted when comparing the four groups of animals. In Fig. 2, the peptidase activities (aminopeptidase N, angiotensin-converting enzyme, and neutral endopeptidase) in brain regions of untreated NEP + / + and NEP − / − mice are compared. Fig. 2A focuses on the three enzyme activities in membrane preparations of the tegmentum/colliculi, the region containing the ventral tegmental area, which is known to be involved in the pathogenesis of alcohol addiction (Herz, 1997; Spanagel et al., 1992). As expected, the neutral endopeptidase-deficient mice did not display any neutral endopeptidase activity. Fig. 2B and C present the results of the test, whether the lack of neutral endopeptidase may induce or influence the activities of the two related enzymes in different brain regions. Minor (but statistically significant) differences between the wild types and the knockout mice were detectable for angiotensin-converting enzyme only in the cortex and for aminopeptidase N in the cortex and thalamus. We conclude that these small differences do not indicate a uniform tendency towards a compensation for the loss of neutral endopeptidase activity by induction of angiotensin-converting enzyme or aminopeptidase N activity.

Furthermore, we investigated whether the complete loss of neutral endopeptidase in the knockout mice resulted in a marked delay in the degradation of enkephalin in the central nervous system (CNS) of these animals. As can be derived from the K_m-values in Fig. 3, enkephalin is mainly degraded by aminopeptidase N, and as described above, the activity of aminopeptidase N was similar in the NEP + / + and NEP − / − animals. Therefore, we did not find significant differences in enkephalin degradation between NEP + / + and NEP − / − mice. An example (striatum) is shown in Fig. 4. If aminopeptidase N was blocked by 10^{-4} M bestatin or amastatin, the degradation of enkephalin was strongly reduced. After incubation for 1 h, enkephalin degradation was generally reduced by 60–80% in comparison to that in the absence of aminopeptidase inhibitors. Under these conditions, the enkephalin degradation in most of the investigated brain regions of NEP − / − mice was smaller than in the corresponding regions of NEP + / + mice, although a statistically significant difference ($P < 0.05$) was only observed in the striatal region (Table 1). Although these results do not show any differences in the degradation of enkephalin between NEP + / + and NEP − / − animals, they may not reflect the in vivo situation. Much of the aminopeptidase N activity in brain is associated with blood vessels (Hersh et al., 1987) and

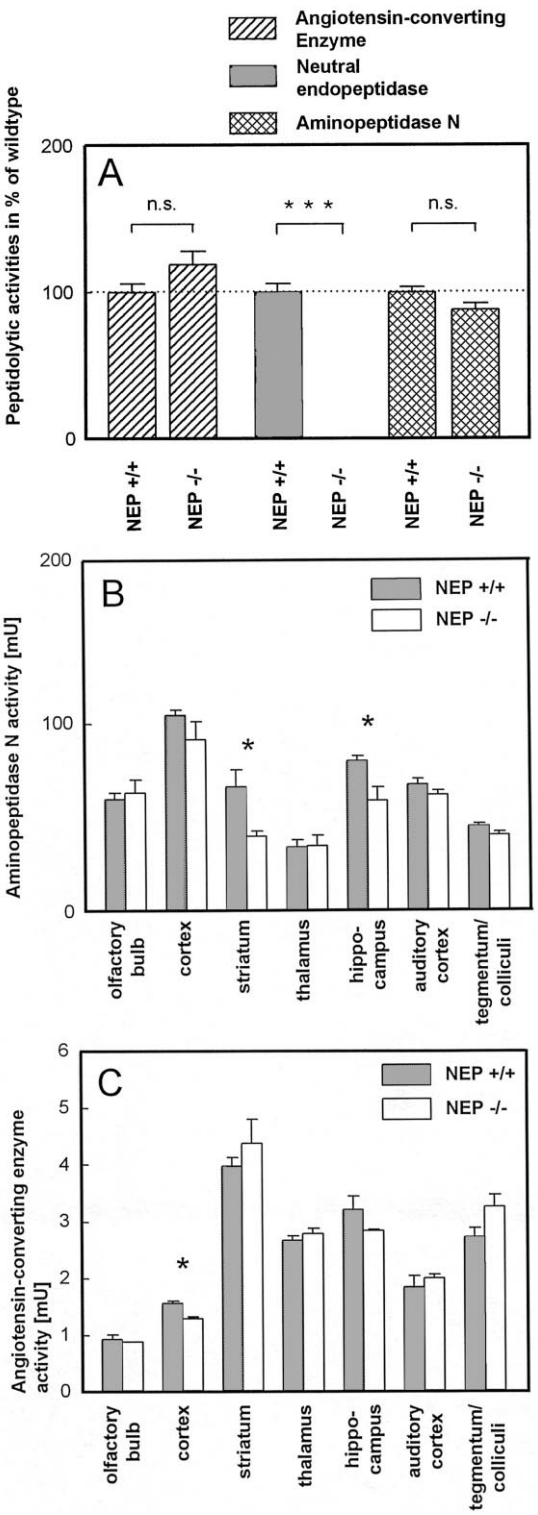

Fig. 2. Aminopeptidase N, neutral endopeptidase and angiotensin-converting enzyme activities in the brain of NEP+/+ and NEP−/− mice. Enzyme activities were measured as described in Section 2 (mean values ±S.E.M., $n = 15$). The significance of differences was calculated by two-sided t test $^{***}P < 0.001$, $^{*}P < 0.05$. (A) Relative activities of aminopeptidase N, neutral endopeptidase, and angiotensin-converting enzyme in tegmentum/colliculi. (B) Aminopeptidase N activity in mU (nMol TyrAMC degradation/min/mg protein) in distinct brain regions. (C) Angiotensin-converting enzyme activity in mU (nMol His-Leu formation/min/mg protein) in distinct brain regions.

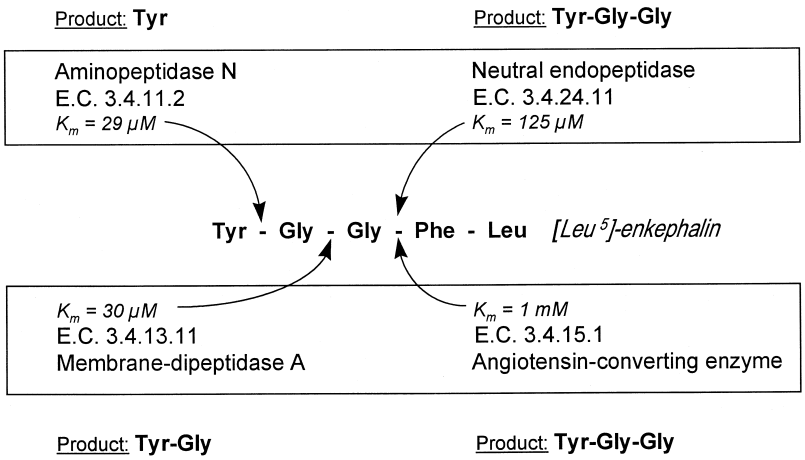

Fig. 3. Enzymes involved in enkephalin degradation in the CNS of mice.

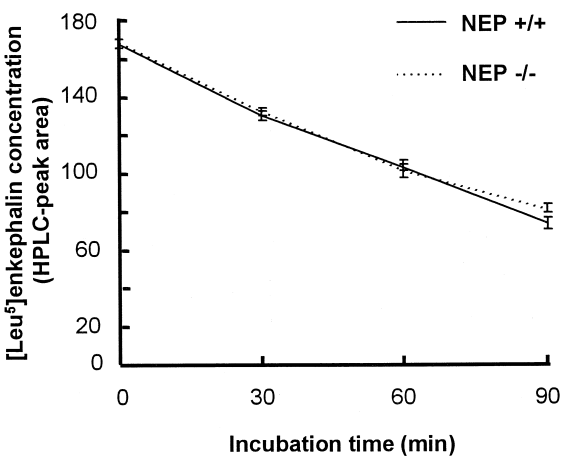

Fig. 4. [Leu^5]enkephalin degradation by striatal membrane preparations from NEP$-/-$ and NEP$+/+$ mice. The Leu-enkephalin concentration was quantified by HPLC as described in Section 2 (mean peak areas $\pm$ S.E.M.; $n = 3$). At the start of the experiment, the [Leu^5]enkephalin concentration in the incubation medium was 10^{-4} M, and the protein concentration was 0.5 mg/ml.

would not be available for degrading enkephalins at enkephalinergic synapses.

Fig. 5 presents the angiotensin-converting enzyme activity in cortex and olfactory bulb after 4 weeks of voluntary alcohol consumption. In these two regions, alcohol induced elevations of angiotensin-converting enzyme activity were detectable in NEP $-/-$ as well as NEP $+/+$ mice. In the tegmentum/colliculi, alcohol consumption significantly increased angiotensin-converting enzyme activity in wild type mice (3.24 ± 0.09 in the alcohol group versus 2.73 ± 0.15 in the water group, $P < 0.05$), but had no effect in the knockout mice. In all other brain regions, the alcohol-induced alterations of the angiotensin-converting enzyme activity were not significant.

A short period of alcohol consumption led to reduced neutral endopeptidase activities in distinct regions of the CNS (olfactory bulb: 0.364 ± 0.012 versus 0.393 ± 0.025, $P < 0.05$; cortex: 0.280 ± 0.021 versus 0.309 ± 0.025, $P < 0.05$) in the wild type mice. These alcohol dependent

Table 1
[Leu^5]enkephalin degradation by membrane preparations of different brain regions from neutral endopeptidase gene knockout mice (NEP $-/-$) and their wild-types in presence of the aminopeptidase inhibitor bestatin (10^{-4} M). After 2 h of incubation, the percentage of degraded enkephalin was quantified by HPLC (mean $\pm$ S.E.M; $n = 3$). At the start of the experiment, the enkephalin concentration in the incubation medium was 10^{-4} M and the protein concentration was 0.5 mg/ml

	[Leu^5]enkephalin degradation by brain homogenates after 2 h in presence of 10^{-4} M bestatin (in percentage of initial concentration)		
	NEP $+/+$ (%)	NEP $-/-$ (%)	Significance
Olfactory bulb	15.4 ± 2.0	8.1 ± 2.9	n.s.
Cortex	11.3 ± 1.6	7.9 ± 0.3	n.s.
Striatum	18.7 ± 1.2	11.3 ± 1.9	$P < 0.05$
Thalamus	18.4 ± 2.1	9.5 ± 2.8	n.s.
Hippocampus	11.0 ± 1.4	8.3 ± 1.7	n.s.
Auditory cortex	22.6 ± 2.3	24.7 ± 0.3	n.s.
Tegmentum/colliculi	18.4 ± 0.9	13.2 ± 3.3	n.s.

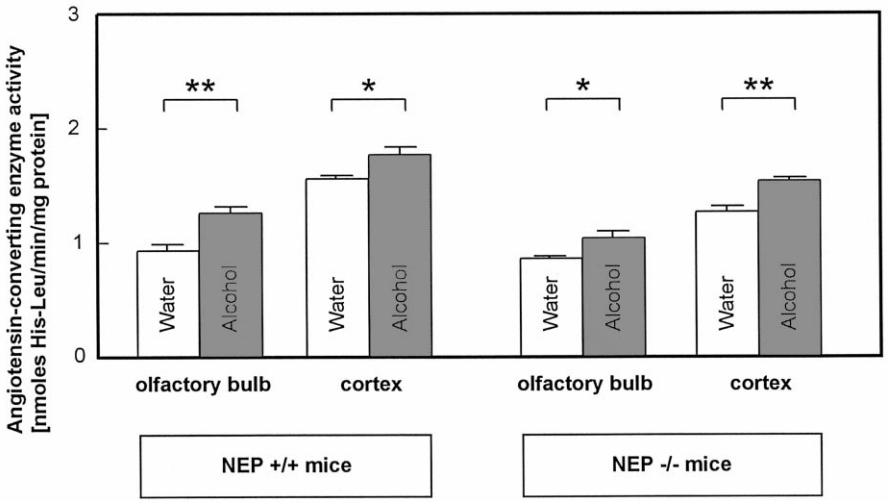

Fig. 5. Alteration of angiotensin-converting enzyme activity (measured as described in Section 2) by voluntary alcohol consumption over 4 weeks. Alcohol: free choice between 10% alcoholic beverage and water; Water: only water; mean ± S.E.M., $n = 15$; $^{*}P < 0.05$, $^{**}P < 0.01$, by two-sided t test.

alterations of neutral endopeptidase activity correspond well to results of earlier experiments, in which alcohol preferring C57BL/6 mice were offered alcohol for a short period of time (Winkler et al., 1998). The aminopeptidase N activity was not significantly altered after alcohol consumption (data not shown).

4. Discussion

The absence of any compensation for the loss of neutral endopeptidase by functionally and structurally related peptidases (aminopeptidase N, angiotensin-converting enzyme) in the untreated animals shows that neutral endopeptidase-deficient mice (NEP −/−) and the corresponding wild type (NEP +/+) constitute an excellent animal model to characterize neutral endopeptidase-relevant processes. On the other hand, we cannot exclude that other membrane components are differently expressed in the two genotypes.

NEP −/− mice showed no significant changes in their total fluid consumption, but drank about 40% more alcohol than NEP +/+ mice. The elevated alcohol consumption in NEP −/− mice must be discussed in relation to both the measured tissue enkephalin levels (Saria et al., 1997) and enkephalin outflow studies (Saria et al., 1999) performed in these animals. The reduced tissue enkephalin levels in distinct CNS regions of neutral endopeptidase deficient mice (striatum and hypothalamus) were postulated to result from modifications in biosynthesis (Saria et al., 1997). These data cannot automatically be equated with appropriate levels of extacellular enkephalins, but appear, at first sight, to support the ''opioid deficit theory''. According to this theory, a deficiency of opioids in some brain regions is associated with an elevated risk of alcohol disease in man and with a preference for alcohol in rats and mice (Sandi et al., 1990a,b; Iukhananov et al., 1993; Ng et al., 1996). The peptidolytic degradation of [Leu^5]enkephalin was found comparable in the two groups of alcohol-naive animals. However, it must be considered that degradative studies using isolated membrane preparations of brain regions only partly represent in vivo metabolism. Cellular compartmentalization and localization are lost in such experiments. Thus we cannot absolutely exclude that enkephalin metabolism at enkephalinergic synapses differs between the NEP −/− and NEP +/+ mice. Moreover, most recent studies revealed comparable basal enkephalin release from striatal slices of NEP −/− mice and NEP +/+ mice. However, K^+ stimulated [Leu^5]enkephalin outflow was significantly higher in the knockout mice (Saria et al., 1999). This report suggests inducable local elevations of enkephalin levels in the neutral endopeptidase gene knockout mice, as was originally expected for these animals and even proposes a basis for discussions contradictory to the deficit theory (''Surfeit Hypothesis'', Reid and Hunter, 1984; Hubbell et al., 1987; Stromberg et al., 1997). We postulate that further natural substrates of neutral endopeptidase, such as neuropeptide Y, may be involved in the neutral endopeptidase-alcohol relationship. The importance of neuropeptide Y in alcohol consumption has also been discussed recently (Thiele et al., 1998). The brain levels of these neuropeptides and their corresponding release rates are still unknown for NEP −/− and NEP +/+ mice.

Furthermore, we cannot exclude that the elevated alcohol consumption in NEP −/− mice is at least in part calorie driven, as the two genotypes significantly differed in their food intake. Possibly, this indicates a higher energy requirement in these animals. The total fluid consumption did not differ between the two groups. Consequently, no antidiuretic effect of enkephalins, as described by Tsushima et al. (1986) after enkephalin microinjections into hypothalamic regions, was evident.

Previous results showed interactions between alcohol intake and the activity of peptidolytic enzymes. Besides the reduction of alcohol consumption by selective inhibition of peptidases (Grupp 1992; Grupp and Chow, 1992; Grupp et al., 1991; Szczepanska and Grupp, 1993; Szczepanska et al., 1996a,b), we found that chronic alcohol consumption alters the activity of peptidolytic enzymes in several regions of the CNS (Siems et al., 1997; Winkler et al., 1998). An alcohol-dependent effect was found in several animal models. Whereas long-lasting alcohol consumption in free choice models (up to 2 years in rats, >3 months in mice) resulted in highly significant elevations of peptidase activities (especially neutral endopeptidase), the effects of shorter alcohol treatment (up to 4 weeks) were non-uniform. A 4-week treatment without final withdrawal of alcohol even led to reduced neutral endopeptidase activity and expression in some brain regions (Winkler et al., 1998). Based on this knowledge, we have now tested whether the loss of an important neuropeptidase in the NEP $-/-$ mice would influence the alcohol-induced modulation of the related peptidolytic enzymes. The results indicate that the loss of neutral endopeptidase is of negligible influence on the modulation of other peptidolytic enzymes by alcohol and agree with our previous findings (Winkler et al., 1998). As already described above for the alcohol-naive animals, the gene targeting had very distinct neutral endopeptidase-related effects but no significant compensatory or modulatory consequences for the other peptidases.

Summarizing our results, we showed that the total loss of the neutral endopeptidase is not compensated by functionally related peptidases such as angiotensin-converting enzyme or aminopeptidase N. Whether the elevated alcohol consumption in the NEP $-/-$ mice is connected to altered enkephalin levels in distinct CNS regions or with an elevated enkephalin outflow or whether other neuropeptides are involved in this regulation should be investigated in future experiments.

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