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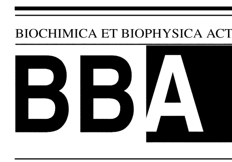

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Gene expression and activity of specific opioid-degrading enzymes in different brain regions of the AA and ANA lines of rats

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Abstract

There is increasing evidence that alcoholism runs in families suggesting that genetic factors may play a role. In support of this hypothesis, the alcohol-preferring (AA) and the alcohol-avoiding (ANA) rat lines have been developed through selective outbreeding. Numerous studies indicate that the endogenous opioid system may be involved in controlling ethanol consumption. Changes in opioid peptides and opioid receptors have been described after ethanol intake. But, the influence of ethanol on peptidolytic degradation of opioid peptides has been largely ignored, although the peptidase-mediated metabolism of neuropeptides is known as an important regulatory site of peptidergic transmission. Neutral endopeptidase 24.11 (NEP) and angiotensin-converting enzyme (ACE) degrade neuropeptides, including enkephalin and are expressed in the brain. Furthermore, a good correspondence between the regional distribution of NEP and opioid receptors in rat brain has already been reported pointing to a possible role of NEP in regulating opioid peptides. For both enzymes studied, the gene expression pattern was found to be in good agreement with the corresponding enzyme activities in the brain regions investigated, showing the highest levels for both specific mRNAs and enzyme activities in the striatum. Differences in both measured parameters were detected in distinct brain regions of AA and ANA rats. Furthermore, in some brain regions discrepancies between ACE and NEP mRNA levels and the corresponding enzyme activities were observed. For example, in olfactory bulb and striatum such discrepancies were found for both enzymes studied. In tegmentum/colliculi a higher NEP gene expression in AA rats was associated with a higher NEP enzyme activity compared to the amounts found in ANA rats. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Alcohol; Endogenous opioid peptides; Enkephalin; Neutral endopeptidase; Angiotensin-converting enzyme; Brain

1. Introduction

Epidemiological and experimental studies indicate that genetic factors are involved in the predisposition to and in the development of alcoholism [1,2]. Selective breeding leading to alcohol-preferring and -non-preferring lines of animals supports this hypothesis. The alcohol-preferring (AA) and the alcohol-avoiding (ANA) rat lines, selected for differential alcohol drinking since 1960's, provide an excellent tool for the identification of physiological and biochemical

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parameters related to the regulation of alcohol drinking [3]. It is generally accepted that multiple genes are acting in concert to either increase or decrease an individual's vulnerability to alcoholism [4,5]. Indeed, a number of different neurotransmitter and neuromodulator systems are known to be influenced by ethanol [6,7]. Among these the endogenous opioid system is considered to be important in mediating some of the alcohol's effects [4]. Animal and human studies have shown that treatment with opioid antagonists can reduce ethanol consumption [8–10], and differences in both the density of μ and δ opioid receptors as well as in the levels of opioid peptides between the AA and ANA rats have been shown [11–13]. From these studies it seems likely, that more than one component of the endogenous opioid system is involved in controlling ethanol consumption.

Homeostasis of a system depends on its components, their interaction and regulation by biosynthesis, processing and degradation. Although the termination of peptidergic transmission includes receptor-mediated internalization of neuropeptides, hydrolysis of peptides by peptidases is accepted to represent a major mechanism for terminating the peptidergic signal [14,15]. In regard to the opioid system the NEP plays a key role in the inactivation of the opioid peptide enkephalin, an endogenous ligand of opioid receptors, in the brain [16]. Furthermore, a parallel distribution of NEP and μ and/or δ opioid receptors in different rat brain regions has been reported [17]. Enzyme inhibitors have been shown to attenuate ethanol consumption and in mice with a high preference for ethanol an enhanced enkephalin degradation in striatum has been detected [18,19]. Long term treatment with ACE inhibitors leads also to a reduction in voluntary ethanol intake by animals, but the underlying mechanism of this effect is much less understood [37]. However, the enzyme is an integral membrane peptidase that degrades among other neuropeptides enkephalin and substance P in vitro and exhibits enrichment at synaptic junctions [15]. Recently, the brain ACE was shown to be involved in central nociceptive mechanisms by interaction with the endogenous opioid system [20].

Regulation of peptide-mediated signals by peptidases suggests that these enzymes may, in turn, be regulated by other signals. So far, there are only a few studies describing peptidase regulation: Phorbol diesters change the NEP expression in rabbit synovial fibroblasts [21] and dopamine receptor agonists and antagonists alter the gene expression of the neuropeptide processing enzymes PC1 and PC2 in rat pituitary [22]. It is not known whether ethanol can affect extracellular peptide levels in the brain via alterations in corresponding peptidase metabolism. But, studies describing an effect of NEP and ACE inhibitors on ethanol consumption point to a role of these peptidases in mediating some of ethanol's actions.

Thus, the objective of the present study was to investigate the activity and gene expression of NEP and ACE in different brain regions of the AA and ANA lines of rats under alcohol-naive conditions. The activity of NEP and ACE was determined by HPLC analysis, whereas a quantitative RT-PCR approach allowed determination of the mRNA level of those enzymes.

2. Materials and methods

2.1. Animals and dissection

Adult (10 weeks old) male AA and ANA rats (Natl. Public Health Inst., Helsinki, Finland) from the 62nd generation were housed and handled for 4 weeks in groups of two on a 12/12 h light/dark cycle with food and water available ad libitum. These animals had never had any contact with alcohol. Ten rats of each line were used for biochemical analysis and two rats of each strain were used for the evaluation of molecular-biological data.

Animals were killed by decapitation. The brains were rapidly removed and dissected on chilled petri dishes to obtain the brain regions (thalamus, hippocampus, bulbus olfactorius, striatum, auditory cortex (including amygdala) and tegmentum/colliculi (including ventral tegmental area and substantia nigra)) as previously described [23]. The tissues were frozen on dry ice and stored at −80°C until use. For determination of the enzyme activities brain regions had to be pooled as followed: the olfactory bulb and hippocampus from five animals were used for one membrane preparation and the striatum, thalamus, auditory cortex and tegmentum/colliculi from 3 or 4 animals were used for one membrane preparation.

2.2. Membrane preparations

Membrane preparations were done according to the procedure described [24]. In brief, tissues were homogenized using a glass–teflon homogenizer in ice cold 50 mM Tris buffer (pH 7.4), filtered through nylon gaze and centrifuged for 20 min at 4°C and $43,000 \times g$. The remaining pellet was resuspended in the same buffer. The suspension was incubated for 30 min at 24°C in a shaking water bath in order to degrade interfering endogenous opioids and then it was centrifuged again. The final pellet was again resuspended in buffer, containing 0.32 M sucrose and stored in portions until use at −80°C. The protein concentrations of the membranes were measured according to the method of Bradford [25].

2.3. Determination of NEP and ACE activities

After centrifugation membranes were resuspended in a 50 mM Tris-buffer (pH 7.4). 100 μM leu-enkephalin (LENK) was employed to measure NEP and ACE activities. Parallel assays were run with D-ala^2-leu-enkephalin (DALEK) (100 μM) as a substrate in order to validate the measured NEP activities. The following enzyme inhibitors were used for inhibition of the accompanying enzymes: 10^{-4} M bestatin (Sigma, Deisenhofen, Germany) for inhibition of aminopeptidases, 10^{-6} M lisinopril (Merck-Sharp-Dohme, USA) for inhibition of ACE and 10^{-6} M phosphoramidon (Sigma, Deisenhofen, Germany) for inhibition of NEP. A mixture of bestatin with lisinopril was used to yield NEP activity and for the measurement of ACE activity the inhibitors bestatin and phosphoramidon were used. For determination of the ACE activities membrane preparations contained 1.5 mg protein/ml, whereas for the NEP measurements a concentration of 0.5 mg protein/ml was used. 50 μl of membrane preparation, substrates and inhibitors were incubated in a final volume of 250 μl at 37°C for 30, 60 and 90 min, respectively. The reactions were stopped by addition of 1/10 volume of 0.35 M $HClO_4$ at 0°C. The measurement of NEP and ACE activity was carried out by product formation (the tripeptides Tyr–Gly–Gly, respectively, Tyr–D-Ala^2–Gly) with HPLC (Shimadzu) using an isocratic elution system (buffer consists of 0.15 M $NaClO_4$ and 0.01 M NaH_2PO_4, pH 2.2 with 4 or 6% acetonitril). For separation a Nucleosil C-18 column was used. The detection was done at 216 nm. The enzyme activities were calculated from the areas of the peaks of the corresponding degradation products and were expressed as nmol tripeptide/min/mg protein.

2.4. RNA isolation and DNase treatment

Total RNA was prepared from tissue by a single step method using Trizol (Gibco BRL, Eggenstein, Germany) according to the manufacturer's instructions. The yield and purity of the RNA was determined spectrophotometrically by UV absorption at 260 nm.

Before quantitative RT-PCR all samples were treated with RNase-free DNase (1 μl/μg total RNA) (Promega, Heidelberg, Germany) for 30 min at 37°C. The reaction was stopped by heat treatment for 10 min at 70°C and the RNA was then purified by phenol/chloroform treatment. Effectiveness of the DNase treatment was routinely checked by a PCR reaction without reverse transcription.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR for NEP was carried out using the EZ rTth RNA PCR kit (Perkin Elmer, Weiterstadt, Germany). 200 ng of total RNA were amplified by RT-PCR in a final volume of 50 μl $1 \times$ EZ buffer, including 300 μM of each deoxy-NTP, 2.5 mM $Mn(OAc)_2$, 5 U rTth DNA Polymerase, 10 pmol of the biotinylated 5′-NEP primer and 10 pmol of the 3′-NEP primer. The mixture was overlaid with 15 μl chill-out-wax (Biozym, Hess. Oldendorf, Germany). Following the RT-reaction at 58°C for 30 min the amplification profile consisted of an initial cycle of 2 min at 95°C, then 40 cycles of the following protocol: denaturation at 94°C for 45 s, primer annealing at 55°C for 45 s and extension at 72°C for 1 min for the first cycle with an extension time of 1 s per cyclus. During the last cycle the primer extension lasted 7 min.

RT-PCR for ACE was carried out using the standard RT-PCR protocol of the Perkin Elmer RNA PCR Kit. For each reaction 200 ng total RNA were used. Reverse transcription was carried out using 50 pmol random hexamers per reaction, in the PCR 10 pmol of the biotinylated 5′-ACE primer and 10 pmol

of the 3′-ACE primer were used. The PCR profile consisted of an initial cycle of 2 min at 95°C, then 40 cycles of the following protocol: denaturation at 94°C for 45 s, primer annealing at 67°C for 45 s and extension at 72°C for 1 min for the first cycle with an extension time of 1 s per cyclus. During the last cycle the primer extension lasted 7 min.

For quantification of the gene expression RNA standards were diluted prior to use in water and either known amounts of both RNA standards (for standard curve) or 200 ng of total RNA with a known amount of mutated RNA standard were mixed together.

Primers were constructed from the published cDNA sequences of rat ACE gene [26]. The sequences of the primers were biotin-5′-CGGCAAGGACTTCAG-GATCAAGC-3′ (sense) 5′-CACTGCTGAGCAGGT-TGAGACTG-3′ (antisense).

Primers were constructed from the published cDNA sequences of rat NEP gene [27]. The sequences of the primers were biotin-5′-AATCAGCTGCTCGACT-GATCC-3′ (sense) 5′-TTTTGCTTTCTGCACTGC-TAC-3′ (antisense).

2.6. Preparation of RNA standards

The PCR-fragments of the NEP and ACE genes obtained by RT-PCR with RNA isolated from rat striatum were cloned in a PCR II vector (invitrogen, Leek, Netherlands) as described by the manufacturer. The mutated standard was created using the Chameleon double-stranded, site-directed mutagenesis kit (Stratagene, Heidelberg, Germany) in such a way that the mutated standard differed from the sample DNA sequence by only one nucleotide.

The sequence of the mutation primer for ACE was 5′-CCACCACGAAATGGGCCA**G**ATCCAGTATT-TCATGC-3′. The C ⇒ G point mutation is indicated boldprinted in the sequence.

The sequence of the mutation primer for NEP was 5′-CGCAATGTCATCCCTGA**C**ACCAGTTCCC-GATACAG-3′. The G ⇒ C point mutation is indicated boldprinted in the sequence.

Plasmid DNAs of the sample and mutated sequence were linearized with a restriction enzyme that cleaves downstream of the inserted fragment. RNA standards were prepared from these linearized plasmids by in vitro transcription. The RNA was diluted to 10^{11} molecules/μl in water containing 1 U/ml RNasin (Promega, Heidelberg, Germany) and stored at 4°C until use. At these conditions RNA preparations were stable for about 3 weeks.

2.7. Quantification by solid-phase minisequencing

Quantification was done as described previously [28]. A scheme of the assay is shown in Fig. 1. 1/10

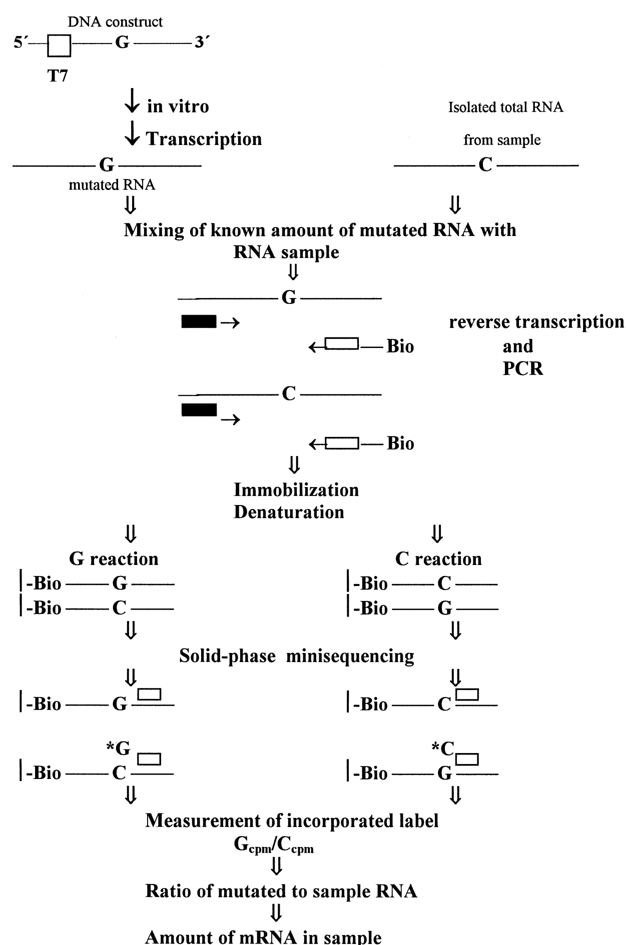

Fig. 1. Principle of the RT-PCR and solid-phase minisequencing method. A mixture of the mutated and sample RNA is reverse transcribed and amplified using one biotinylated and one unbiotinylated PCR primer. The PCR product is captured in streptavidin-coated microtitration wells and denatured. The nucleotide that differs between the two sequences is detected in the captured DNA strand by two separate minisequencing reactions. In these reactions a detection step primer annealing immediately 3′ of the variable nucleotide is elongated by TAQ DNA polymerase with one single [^{3}H]-labeled NTP complementary to the nucleotide to be detected. The ratio of the incorporated labels reflects the initial ratio of the two RNA sequences [31].

of the RT-PCR reaction was analyzed per minisequencing reaction. For one assay every sample was loaded in parallel.

2.8. Statistics

All data were analyzed with Student's *t*-test for independent samples.

3. Results

A quantitative RT-PCR approach for the detection of gene expression was chosen because (i) the material from brain regions of individual rats was limited and (ii) expression of several genes should be determined in parallel from the same material. The RT-PCR technique allows sensitive detection of mRNA species in a very low amount of starting material. However, due to several factors direct quantification of specific mRNA species using this approach is not possible [29]. The most accurate quantitation of RNA can be obtained by competitive RT-PCR, where an internal standard should be coamplified with the sample of interest in the same reaction. Because the efficiency of the PCR depends on the primers as well as both the sequence and the size of the template, the internal standard should be as similar as possible to the sample of interest [30]. In our assays the internal standard (mutated RNA) differed in sequence from the sample RNA only by one nucleotide. RT-PCR is performed with the sample RNA and a known amount of standard RNA, and then the ratio between the two coamplified sequences is determined by the solid-phase minisequencing method. The results allow determination of the absolute amount of investigated mRNA species present in the samples tested [28,31].

First, a standard curve was prepared by mixing 10^6 molecules of the standard RNA with mutated RNA standard in ratios ranging from 0.01 to 10. Plotting of the G_{cpm}/C_{cpm} ratio obtained in the minisequencing test resulted in a linear standard curve (Fig. 2). The G_{cpm}/C_{cpm} ratio is affected by some factors like specific activities of the [^{3}H] NTP's used, specificity of the DNA polymerase and a low amount of background caused by misincorporation of [^{3}H] NTP. Therefore, we used a standard curve in parallel with each set of samples.

ACE mRNA levels and enzyme activities are shown in Fig. 3a and b, respectively. Gene expression data and enzyme activities for the NEP are shown in Fig. 4a and b, respectively. A comparison of the specific mRNA molecules/μg total RNA revealed a significant higher mRNA level for ACE in some brain regions compared to the NEP mRNA levels in the corresponding brain regions (Fig. 3a and Fig. 4a). In striatum and tegmentum/colliculi between 2 and 5 times more ACE mRNA compared

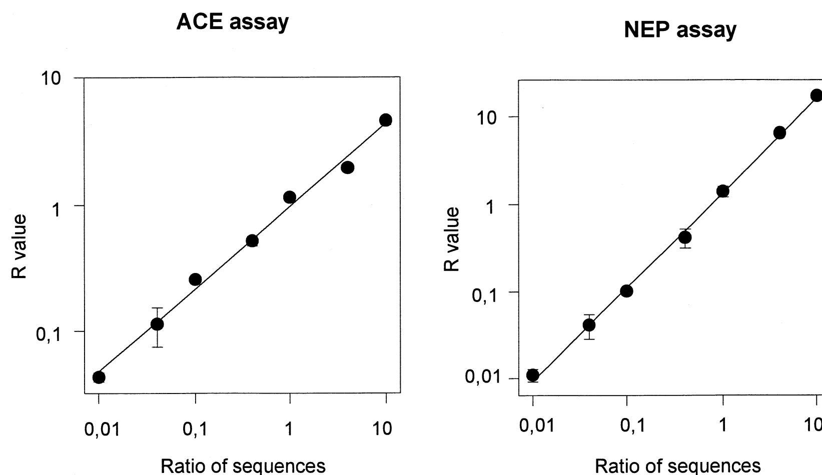

Fig. 2. Standard curves obtained by quantitative RT-PCR for ACE and NEP. R value = ratio of the cpm values obtained in the reaction for detection of the mutated RNA sequence to that in the reaction for detection of the sample RNA sequence. The mean value and variation range (SD) for three separate assays are shown.

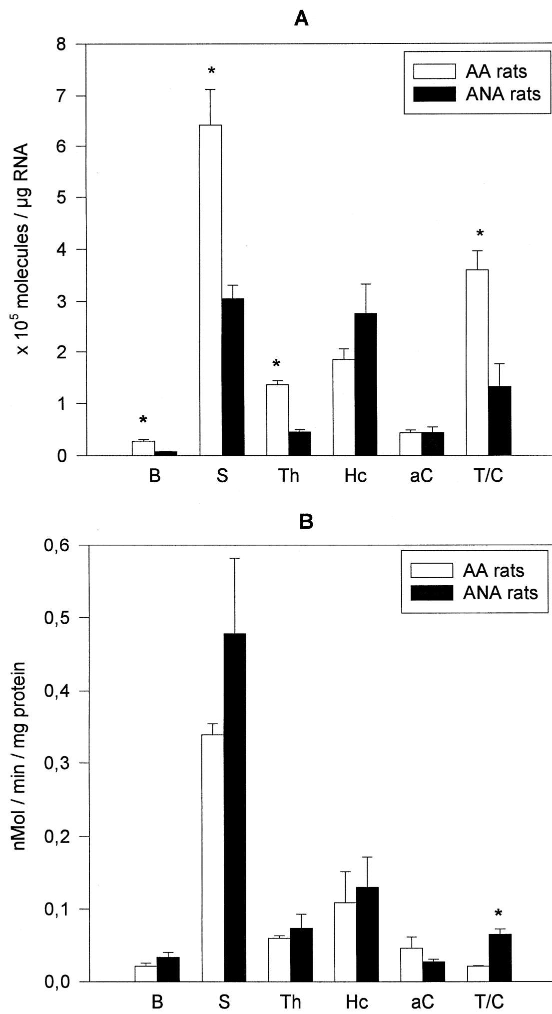

Fig. 3. Gene expression (A) and activity (B) of ACE in brain regions of AA and ANA rats. Gene expression was measured by quantitative RT-PCR ($n = 2$), enzyme activity was determined by degradation of leu-enkephalin measured by HPLC ($n = 10$). Data represent means ± S.E.M. * Significant differences between the two lines of rats in corresponding brain regions with $p < 0.05$. B, bulbus olfactorius; S, striatum; Th, thalamus; Hc, hippocampus; aC, auditory cortex and T/C, tegmentum/colliculi.

with the NEP mRNA level was detected. In the hippocampus the amount of ACE mRNA was even 10 times higher than the NEP mRNA amount. Interestingly, measurement of the corresponding enzyme activities when enkephalin was used as a substrate showed contrary results (Fig. 3b and Fig. 4b). In all investigated brain regions the NEP activity was more than 2 times higher than the ACE activity. However, comparison of the enzyme activities should consider the very different K_m values for enkephalin (factor 1000 better for NEP). Furthermore, ACE is known to degrade several other peptides than enkephalin which was used as a substrate in this study. This may explain, at least in part, the observed discrepancies between gene expression levels and measured enzyme activities.

For both enzymes studied the pattern of gene expression levels is in good agreement with the corresponding enzyme activities in the brain showing the

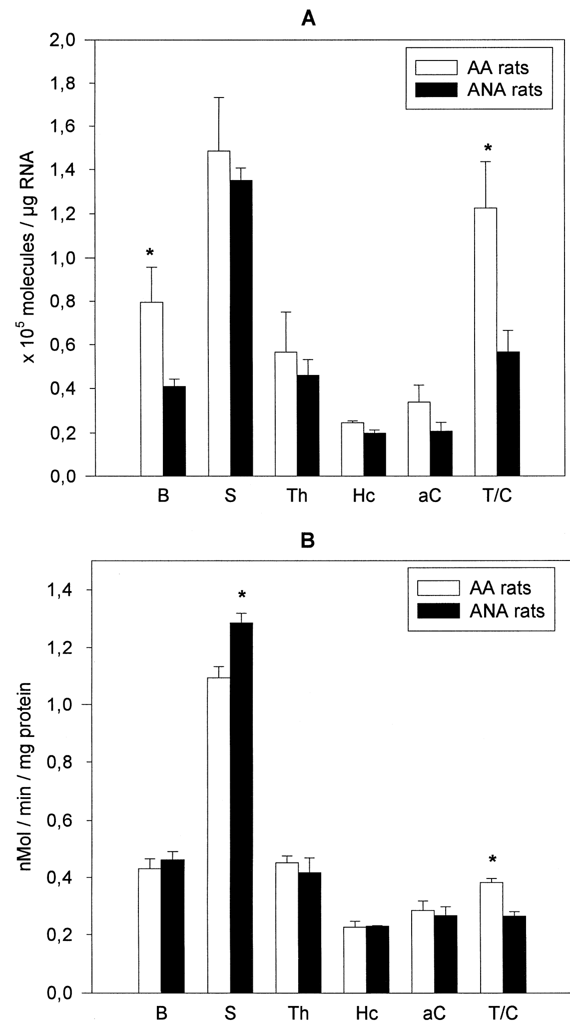

Fig. 4. Gene expression (A) and activity (B) of NEP in brain regions of AA and ANA rats. Gene expression was measured by quantitative RT-PCR ($n = 2$), enzyme activity was determined by degradation of leu-enkephalin measured by HPLC ($n = 10$). Data represent means ± S.E.M. * Significant differences between the two lines of rats in corresponding brain regions with $p < 0.05$. B, bulbus olfactorius; S, striatum; Th, thalamus; Hc, hippocampus; aC, auditory cortex and T/C, tegmentum/colliculi.

highest amounts in striatum. Furthermore, differences in the measured parameters were also detected between the two lines of rats. The gene expression studies of ACE revealed a significantly enhanced mRNA level in olfactory bulb, striatum, thalamus and tegmentum/colliculi of AA rats, whereas for the corresponding ACE enzyme activities an opposite tendency was found. In tegmentum/colliculi of AA rats the ACE enzyme activity was even significantly lower compared to ANA rats. For NEP the same discrepancy was observed in olfactory bulb and striatum, but a good correlation between gene expression and enzyme activity could be detected in other brain regions tested. Interestingly, in tegmentum/colliculi of AA rats a higher NEP gene expression was associated with a higher NEP activity compared to the levels found in ANA rats.

4. Discussion

During the past decades evidence has been accumulating that genetic factors are involved in the predisposition to alcoholism [1,2]. Rodent lines exhibiting innate differences in alcohol preference are used for identifying physiological and biochemical parameters linked to alcohol preference or nonpreference [33]. Numerous studies suggest that the endogenous opioid system is one of the systems involved in genetically determined drug consumption. Opioid receptor antagonists have been shown to decrease ethanol self-administration in several experimental paradigms [4] and specific opioid receptor types have been suggested to be important for this effect [34]. These experiments have led to the hypothesis that the positive reinforcing effects of ethanol are in part mediated by the opioid system. Indeed, differences in components of this system have been detected in distinct brain regions of alcohol-preferring and alcohol-nonpreferring animals. Autoradiographic studies showed a significantly higher density of μ-opioid receptors in some brain regions of AA rats compared to the corresponding regions of ANA rats [11]. These areas included regions of the limbic system which are thought to be particularly involved in the reinforcing effects of drugs of abuse. Differences were seen in the nucleus accumbens, the caudate and the ventral tegmental area (VTA). Dopaminergic neurons in the VTA project to the nucleus accumbens as well as to other mesocortical and mesolimbic areas involved in reinforcement. Further evidence suggests that components of the endogenous opioid system may play a role in the regulation of dopaminergic tone [5,35,36].

Differences between AA and ANA rats in opioid peptide levels have also been described [13]. However, there are only a few studies investigating the role of opioid-degrading enzymes in alcoholism. Enkephalin, one of the opioid peptides, is rapidly degraded in the brain by 4 peptidases: aminopeptidase N (E.C. 3.4.17.3.), membrane-dipeptidase (E.C. 3.4.13.11.), NEP (E.C. 3.4.24.11.) and ACE (E.C. 3.4.15.1.) [32]. Whereas the first two enzymes are rather nonspecific, the two latter hydrolyze specifically neuropeptides. Furthermore, the coexistence of NEP and μ- and/or δ-opioid receptors in the brain of mammals has been already described, supporting the hypothesis that NEP has a selective role in the termination of the enkephalinergic signal [17]. NEP inhibitors were shown to attenuate ethanol intake in alcohol-preferring mice which, in addition, were reported to have an increased enkephalin-degrading activity in striatum [18,19]. From animal studies it is known that ACE inhibitors can produce a significant reduction in alcohol consumption and this effect is not the result of an ACE inhibitor-induced alteration in the pharmacokinetics of ethanol [37,38]. Recently, the necessity of ACE inhibitors for the complete protection of exogenous [met]enkephalin from degradation was described [39]. From our results it can be concluded that ACE participates in enkephalin degradation in rat brain regions. The rate of enzymatic degradation of a specific substrate is determined by the K_m- and the V_{max}-value of the enzyme studied. Although the K_m value of ACE for enkephalin is 1000 times less than the K_m value of NEP, the amount of ACE (V_{max}) is high enough to account for 0.2–0.5 times of the enkephalin hydrolysis when compared to NEP (Fig. 3b and Fig. 4b). This is in good agreement with the gene expression data, where 2–10 times more ACE mRNA was measured compared to NEP mRNA levels in different brain regions (Fig. 3a and Fig. 4a).

Nevertheless, it should be noticed that both enzymes degrade also other neuropeptides. Especially, ACE is known to convert preferentially angiotensin I to angiotensin II and to degrade bradykinin as well as

substance P [40–42]. Therefore, the reduction of ethanol intake by ACE- and NEP-inhibitors may in part be mediated through their influence on the degradation of other neuropeptides than enkephalin.

Although in most investigated brain regions of AA rats an enhanced level of ACE and NEP mRNA was found, the corresponding enzyme activities did not show adequate differences (Figs. 3 and 4). This fact was more pronounced for ACE than for NEP. The reason for the differences between gene expression and enzyme activity is still not clear, but differences in post-transcriptional processes and/or a higher turnover as well as inactive forms of the enzymes could be assumed. Similar discrepancies between gene expression data and protein levels has been described for the POMC system in AA and ANA rats. Despite the higher content of POMC mRNA in the hypothalamus of the AA rats, the basal rate of corresponding peptide release was higher in the hypothalamus of the ANA rats indicating an involvement of factors controlling translation and/or transport processes [43].

Interestingly, a higher NEP gene expression was associated with a higher NEP activity in tegmentum/colliculi of AA rats compared to the corresponding values found in ANA rats. The tegmentum/colliculi contains the VTA which is known to have high concentrations of the components of the endogenous opioid system. The higher NEP activity found in tegmentum/colliculi of AA rats could lead to a higher degradation rate of enkephalin in this area. Indeed, a lower content of (leu)enkephalin was reported in the VTA of AA rats compared to the value found in ANA rats [13]. Moreover, activation of dopaminergic neurons in the VTA may be involved in the reinforcing effects of drugs of abuse [44]. Supporting this hypothesis, ethanol self-infusion into the ventral tegmental area by alcohol-preferring rats has been reported [45]. It is proposed that ethanol may exert these effects indirectly via various transmitter systems, including the opioid system [46]. The reinforcing effects of enkephalins are thought to be mediated mainly by δ opioid receptors in the VTA, where an activation of δ opioid receptors causes activation of the mesolimbic dopamine pathway via disinhibition of GABA interneurons. Therefore, the observed differences in the NEP activity between the two lines of rats give further evidence that neurobiological substrates in the VTA may be genetically determined and contribute to differences in ethanol drinking behavior.

In conclusion, the present study demonstrated the first evidence for genetically determined differences in some brain regions between AA and ANA rats in the enkephalin-degrading enzymes ACE and NEP. Further studies comparing gene expression and enzyme activity in the presence and after ethanol treatment will provide more information on the importance of such differences between the AA and ANA rats.

Acknowledgements

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