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Alcohol consumption is controlled by angiotensin II

Bjoern Maul,* Wolf-Eberhard Siems,* Margret R. Hoehe,† Gisela Grecksch,‡ Michael Bader,§ Thomas Walther§,¶

*Institute of Molecular Pharmacology, Berlin, Germany; †Genome Research, Max-Delbrueck-Center for Molecular Medicine (MDC), Berlin-Buch, Germany; ‡Institute of Pharmacology and Toxicology, University of Magdeburg, Magdeburg, Germany; §Hypertension Research, Max-Delbrueck-Center for Molecular Medicine (MDC), Berlin-Buch, Germany; ¶Department of Cardiology and Pneumology, University Hospital, Benjamin Franklin Free University of Berlin, Germany

Corresponding author: Bjoern Maul, Institute of Molecular Pharmacology, Robert-Roessle-Str. 10, 13125 Berlin, Germany. E-mail: maul@fmp-berlin.de

ABSTRACT

Pharmacological manipulation of the renin-angiotensin system (RAS) appears to alter voluntary alcohol consumption in animals. We have now addressed the role of angiotensin II (AII) in alcohol intake by comparing the alcohol-drinking behavior of mice lacking or overexpressing the angiotensinogen gene in a two-bottle choice paradigm. We found that alcohol consumption was directly related to AII levels. Animals harboring a rat angiotensinogen transgene consumed significantly more of the alcoholic beverage than did controls. This effect could be remarkably reduced by spirapril, an inhibitor of the angiotensin-converting enzyme (ACE). However, mice lacking the angiotensinogen gene exhibited a markedly reduced alcohol intake. These results indicate a role for the angiotensinogen gene in alcohol consumption via modulation of AII.

Key words: transgenic mice • ACE inhibition • spirapril

Alcohol abuse is a prototypic complex disease that is determined jointly by multiple genes and environmental influences. By application of transgenic technology, a role in alcohol-related behavior has been suggested for the serotonin 5-HT_{1B} (1) and dopamine D_2 (2) receptor genes, and likewise for the gene encoding neuropeptide Y (3).

The angiotensinogen gene encodes a protein of 477 amino acids, which is cleaved by renin to angiotensin I. The subsequent removal of two amino acid residues by angiotensin-converting enzyme (ACE) produces angiotensin II (AII), which is active, for example, in blood pressure regulation. The potential involvement of the renin-angiotensin system (RAS) in alcohol-consuming behavior in animals has long been suggested, because pharmacological manipulation of the RAS appears to alter voluntary alcohol consumption (4-6). However, the function of AII in this context has remained unclear. Several experiments that demonstrated a significant reduction in alcohol intake by rats upon peripheral application of AII (7,8) contradicted findings suggesting that centrally administered AII may elevate alcohol consumption in rats (4,9) and mice (6). To clarify the role of AII, we tested the correlation between endogenous AII levels and

voluntary alcohol intake in transgenic mice expressing a rat angiotensinogen transgene (TGM123) (10), and in knockout mice lacking the angiotensinogen gene (TLM) (11).

MATERIALS AND METHODS

In situ hybridization

In situ hybridization was performed on formaldehyde-fixed cryostat sections after hydrochloric acid treatment (100 mM) and acetylation in 0.25% (v/v) acetic anhydride in 100 mM triethanolamine buffer, pH 8. The dry sections were incubated overnight with cRNA probes matching the rat and mouse angiotensinogen mRNAs in a hybridization solution containing 50% (v/v) formamide; 10% (w/v) dextran sulfate; 150 mM sodium chloride, 15 mM sodium citrate, pH 7.0; 100 µg/ml salmon sperm DNA; 5× Denhardt solution; 250 µg/ml yeast tRNA; and 100 mg/ml poly(adenylic acid). The probes were synthesized from the vector pRAG 0.3G4 (11) and a pGEM4 (Promega, Mannheim, Germany) derivative harboring a 1-kb *Eco*I/*Bam*HI fragment homologous to exon 2 of the mouse angiotensinogen gene after linearization of the plasmids at appropriate restriction sites. We used the DIG-RNA labeling kit (Roche Biochemicals, Mannheim, Germany) to label the RNA with the hapten digoxygenin. After the washing steps, the slides were treated as recommended by the manufacturer of the labeling kit. The stained sections were covered with Aquatex (Merck, Berlin, Germany) and evaluated under a Nikon microscope.

Preference tests

Mice of the respective genotypes (10,11) were obtained from breeding stocks of M.B. and T.W. at the Max-Delbrueck-Center for Molecular Medicine in Berlin (Germany). The animals were housed in groups of two or three at 22 ± 1°C in a 12 h-12 h light-dark cycle with food and beverage available ad libitum. All animals were habituated to drinking from two tap water bottles over 2 wk, and they were eventually given a free choice between tap water and sucrose solution, quinine solution, or alcohol 24 h a day. The fluid intake during each individual 24-h period was recorded and analyzed statistically. Bottle positions were changed every day.

Locomotor activity

Locomotor activity was tested in an activity meter 46 × 46 cm in size with 15 photo cells in each dimension (MOTITEST, TSE, Bad Homburg, Germany). TGM123 mice (n = 8) and controls (n = 11) were randomized to receive intraperitoneal injections of saline or ethanol at 2.5 g/kg of body weight (20% [v/v] in isotonic sodium chloride solution). The animals were put into the apparatus 10 min after injection. Their activity was monitored for a 10-min period. The values for the travel distance were analyzed statistically by means of SPSS version 8.0 for Windows.

Sleep time

To test the sedative dose sensitivity, TGM123 mice (n = 6) and controls (n = 10) were injected with ethanol at 4 g/kg of body weight (20% [v/v] in isotonic sodium chloride solution). Sleep time was measured as the time after injection required to regain the righting reflex.

Peptidase activitiy

After a 3-wk period of treatment with spirapril at 10 mg/kg of body weight, the animals were killed by decapitation. The brains were rapidly removed and stored until use at −80°C. Membranes were prepared according to the method of Hulme and Buckley (12) and were stored until use at −80°C in a 50 mM Tris buffer containing 320 mM sucrose. ACE activity was measured via a modified fluorimetric method originally developed by Friedland and Silverstein (13). In brief, 10 µl of a membrane preparation was incubated for 30 min with 10 µl of 0.025 M hippuryl-histidyl-leucine as substrate in a chloride-containing phosphate buffer at pH 8.3. The reaction was stopped by addition of 1 ml of 0.4 M sodium hydroxide; fluorescence was developed by a reaction of the produced histidyl-leucine with 100 µl of 2% *o*-phthalaldehyde in methanolic solution. After acidification with 3 M hydrochloric acid and excitement at 365 nm, the fluorescence was measured at 500 nm. We used histidyl-leucine as a standard. Controls of specificity were carried out with 10^{-6} M of the specific ACE inhibitor lisinopril. The total protein contents of the membrane preparations were determined by a Bradford assay (14). Statistical calculations were done by applying a *t* test.

Alcohol degradation kinetics

TGM123 mice and controls ($n = 9$ for each group) were injected intraperitoneally with ethanol at 3.5 g/kg of body weight (20% [v/v] in isotonic sodium chloride solution) at time zero. Samples of 10 µl of blood were taken from the tail tip 120 and 210 min after the injection. The blood samples were processed as described previously (15), and the ethanol contents were measured according to the method of Roach and Creaven (16) in a Perkin-Elmer 8600 headspace gas chromatograph. External standards ranging from 0.5 to 4.0 mg ethanol per ml of blood were run prior to the samples. Statistical calculations were done by applying a two-way repeated measures ANOVA (genotype × time).

RESULTS

Alcohol consumption

To clarify the role of AII in alcohol consumption, we tested the correlation between endogenous AII levels and voluntary alcohol intake in transgenic mice expressing a rat angiotensinogen transgene (TGM123) (10), knockout mice lacking the angiotensinogen gene (TLM) (11), and their respective controls. Whereas TGM123 mice exhibited overexpression of angiotensinogen in the brain, TLM mice were totally devoid of angiotensinogen mRNA (Fig. 1).

TGM123 mice ($n = 10$) and their wild-type littermates ($n = 9$) were held in a free choice paradigm for 7 wk. They were offered the choice between water and 10% (v/v) ethanol. This concentration of alcohol has been shown to provoke aversive responses in mice (17). Indeed, the mice avoided drinking from the alcohol bottle, so that the alcohol preference ratio was smaller than 0.5. Nonetheless, the ratio (Fig. 2*A*) and the alcohol consumption (Fig. 2*B*) were

significantly increased by at least a third in TGM123 mice compared with their wild-type littermates.

Conversely, TLM mice (*n* = 12) showed a smaller alcohol preference ratio than their wild-type littermates (*n* = 12) when being held in a free choice paradigm for 4 wk (Fig. 3*A*). Because mutant mice suffered from severe disturbances in fluid homeostasis caused by defects in their renal glomeruli (18), the total fluid consumption of TLM mice was about twice that of controls (not shown). Nevertheless, these mice drank even substantially less alcohol than did the controls (Fig. 3*B*).

Other tastants

To determine whether TGM123 mice would exhibit a higher affinity to any fluid other than water, we tested them for their intake of a sweet tastant and a bitter tastant in a free choice paradigm. In one experiment, TGM123 mice (*n* = 10) and their wild-type littermates (*n* = 9) were given the choice between tap water and 1.7% (w/v) sucrose in tap water for 6 days. TGM123 mice were not different from controls in their preference for the sucrose solution (Fig. 2C). Therefore, the increased alcohol consumption in TGM123 mice is unlikely to be calorie driven. We also detected no differences between TGM123 mice (*n* = 11) and their wild-type littermates (*n* = 10) in their aversion to bitter beverages when the animals were offered increasing concentrations of a quinine solution versus tap water (Fig. 2*D*).

Influence of spirapril and fluphenazine on alcohol intake

Spirapril, a lipophilic nonpeptidic ACE inhibitory drug (19), was used to test whether impaired sysnthesis of AII would blunt the transgenic effect. An interesting feature of spirapril is its central availability. In fact, brain membrane preparations of treated mice administered 10 mg/kg spirapril via a feeding needle showed a 40.2% reduction in ACE activity; this effect was specific, inasmuch as the inhibition could be blocked by several washing steps (Fig. 4).

After they had been held in the free choice paradigm for 4 wk, TGM123 mice (*n* = 13) and TLM mice (*n* = 12) were given spirapril. Indeed, the substance significantly reduced the elevated preference ratio in TGM123 from 176% to 141% of the mean control value. As expected, spirapril did not reduce the alcohol consumption in TLM mice (not shown).

On the assumption that dopaminergic systems might be involved in the AII-dependent elevation of voluntary alcohol consumption, animals being held in a free choice paradigm were administered fluphenazine at 1 mg/kg of body weight via consumed tap water as calculated from current water consumption. The consumption of alcohol by TGM123 mice was significantly reduced upon administration of the drug, to approach alcohol intake levels observed in the wild-type littermates (Fig. 2*B*).

Sensitivity to central alcohol effects

Experiments were performed to address additional indices of central alcohol action, because changes in alcohol intake may not necessarily be related to the specific pharmacological effects

of alcohol. Compared with controls, TGM123 mice showed a stronger reduction of their horizontal activity in an activity meter during a 10-min test period after application of ethanol at 2.5 g/kg (Fig. 5*A*). Moreover, it took TGM123 mice significantly more time to reestablish their righting reflex after administration of ethanol at 4 g/kg (Fig. 5*B*). These differences in sensitivity to alcohol effects as well as those in alcohol consumption are not due to unequal clearing rates for alcohol, as plasma ethanol concentrations were not different at two time points after injection of the animals with alcohol (Fig. 6).

DISCUSSION

Although the possible involvement of the RAS in alcohol-consuming behavior in animals has long been suggested, the function of AII in this context has remained unclear. We have now demonstrated a direct correlation between endogenous AII levels and voluntary alcohol intake in genetically altered mice. Alcohol consumption in TGM123 mice, which show an elevation of AII levels (10), was significantly increased. Conversely, the alcohol preference ratio of TLM mice lacking the angiotensinogen gene was smaller than that of their controls. In spite of their excessive total fluid consumption, the TLM mice drank even substantially less alcohol than did the controls. Because TLM mice otherwise display completely normal activity in all behavioral paradigms tested (20), we assume that they selectively avoided ingesting the alcoholic beverage in an attempt to compensate the fluid loss caused by glomeruli dysfunction.

The observation that TGM123 mice exhibited increased alcohol consumption is in accordance with findings of Fitts (4), who described increased alcohol intake upon intracerebroventricular infusion of AII in rats. Other authors reported a reduction of alcohol intake induced by exogenously administered AII in rats (7,8). These experiments, however, employed a different mode of administration of the alcoholic beverage. Fitts (4), like us, tested alcohol consumption of animals that had the drug freely available 24 hours a day. In the studies with conflicting results, a limited access procedure was applied. Those animals were given the choice between water and alcoholic beverage for a period of only 40 min, shortly after a pulsatile or bolus application of AII. Moreover, we measured alcohol consumption without exposing the animals to any invasive application procedures. This may provide improved validity of the behavioral parameters investigated. The most likely explanation for the differences between the studies cited is that we and Fitts analyzed animals in which AII levels are changed in the brain. The other authors, however, modified only the systemic levels of the peptide, which is unable to cross the blood-brain barrier. They suggested that systemic AII might act as a short-term satiety signal for alcohol (21), leading to a reduction in alcohol intake. This would be difficult to reconcile in view of the reduction in alcohol consumption upon the suppression of the AII synthesis by ACE inhibitors (22-24). However, inasmuch as most of these drugs cross the blood-brain barrier, they may influence alcohol consumption mainly by decreasing central AII levels, thus eliciting the same effect on alcohol drinking as that observed in TLM mice. Indeed, the ACE inhibitor spirapril, which crossed the blood-brain barrier, suppressed the transgene effect in our experiments. However, the effect of spirapril on ACE did not completely compensate for the AII-induced increase in alcohol consumption. This may certainly be attributed to the incomplete inhibition of the enzyme activity. But even in the case of a total ACE inhibition, alternative pathways for AII synthesis may be unaffected (25).

Brain AII is part of the systems that regulate thirst and sodium intake. This involvement of AII in general thirst regulation could raise the concern that changes in alcohol intake may not be related to the specific pharmacological effects of alcohol. However, TGM123 mice showed a stronger alcohol-associated reduction of their horizontal activity and prolonged sedation at a high alcohol dose. Thus, in these tests, TGM123 mice exhibited a higher sensitivity to alcohol. However, they consumed larger amounts of alcohol in the free choice paradigm. It is interesting to note that mice lacking the D_2 receptor gene were less sensitive to alcohol-induced ataxia than their wild-type littermates while they ingested lower amounts of alcohol in free choice experiments (2). It is known that AII stimulates dopamine release in the brain (26), and angiotensin receptors are abundantly expressed in brain areas such as the nucleus accumbens, where dopaminergic transmission has been strongly implicated in alcohol self-administration and sensitivity (27,28). We found that the voluntary consumption of alcohol of TGM123 mice was significantly reduced upon administration of the dopamine receptor antagonist fluphenazine. Thus, increased alcohol intake in mice overexpressing angiotensin may relate to an interaction of AII with dopaminergic systems.

Taken together, our results shed new light on the connection between AII and alcohol-consuming behavior. They provide a further contribution to the dissection of the genetic architecture of a devastating disease. Recently, a connection was shown between the ACE DD genotype associated with high ACE activity in humans and an increased susceptibility to alcoholism (29). The elucidation of the mechanism of AII action in regulating alcohol-drinking behavior may therefore reveal new pharmacological paradigms for treating alcohol abuse.

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Fig. 1

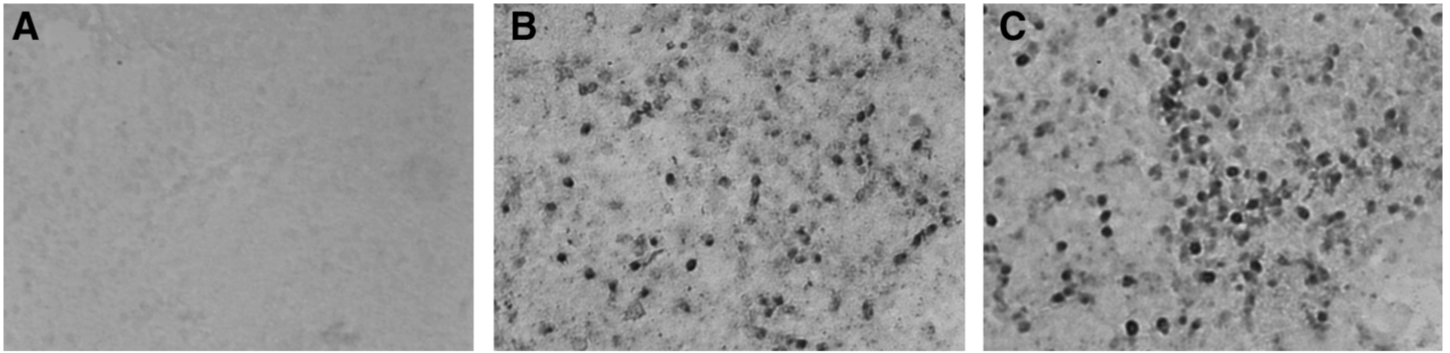

Figure 1. Digital microphotograph of the paraventricular thalamic nucleus (bregma −1.7) at 200-fold magnification after in situ hybridization of mouse brain cryostat sections with angiotensinogen mRNA-specific probes. Sections (14 μm) of the brains of TLM mice **(A)**, TGM123 mice **(C)**, and wild-type littermates of TGM123 mice **(B)** were treated as described in the Materials and Methods section. TGM123 mice and their controls were hybridized with a 1:1 mixture of the rat- and mouse-specific probes. In the case of TLM mice, only the mouse angiotensinogen probe was used. (TLM controls: data not shown.)

Fig. 2

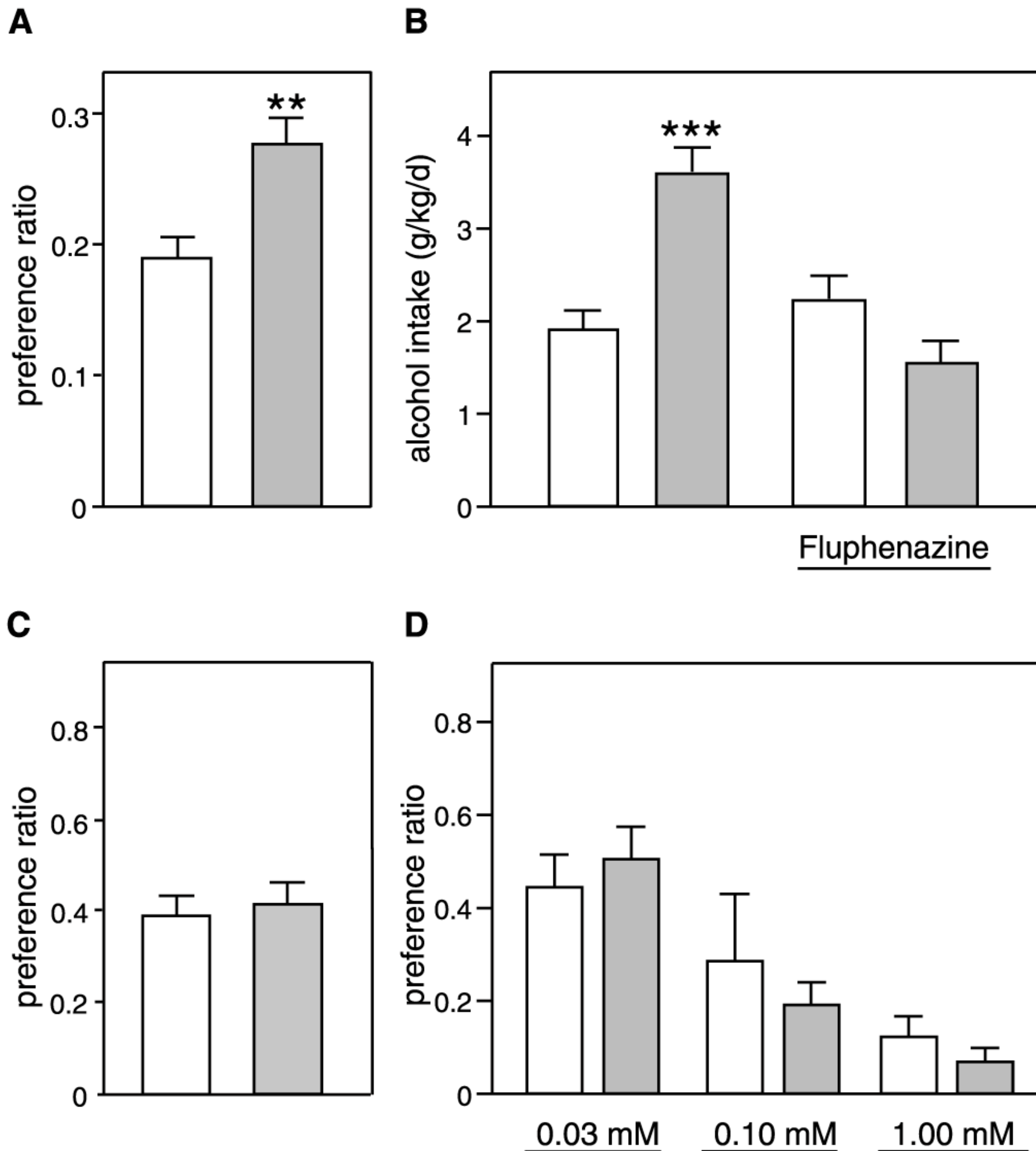

Figure 2. Alcohol consumption in TGM123 mice (filled bars) and their wild-type littermates (open bars) in a two-bottle choice paradigm. All values are means±SE. **A)** Mean alcohol preference ratio [proportion of the 10% (v/v) ethanol solution per total fluid consumption during 32 free choice 24-h drinking periods, $^{**}P<0.01$]. **B)** Influence of fluphenazine on alcohol consumption. Daily alcohol intake is shown for 32 free choice 24-h drinking periods before the administration of fluphenazine (left) and 9 free choice 24-h drinking periods during the administration of fluphenazine (right) (1 mg/kg of body weight). The Bonferroni Multiple Comparison test following significant ANOVA ($P<0.05$) revealed that only the alcohol intake of untreated TGM123 mice was significantly increased compared with the untreated controls ($^{***}P<0.001$). **C)** Mean preference ratio for sucrose [proportion of a 1.7% (w/v) sucrose solution per total fluid consumption during six free choice 24-h drinking periods]. **D)** Aversion to a bitter tastant. Mice were sequentially offered 0.03, 0.1, and 1 mM quinine versus tap water. The preference ratio is shown for four individual free choice 24-h drinking periods. Statistical comparison of data in a two-way ANOVA (concentration × genotype) revealed no significant interaction of genotype and the three different quinine concentrations. The concentration affected the result significantly ($F_{2.20} = 7.55$; $P<0.01$), whereas the genotype did not.

Fig. 3

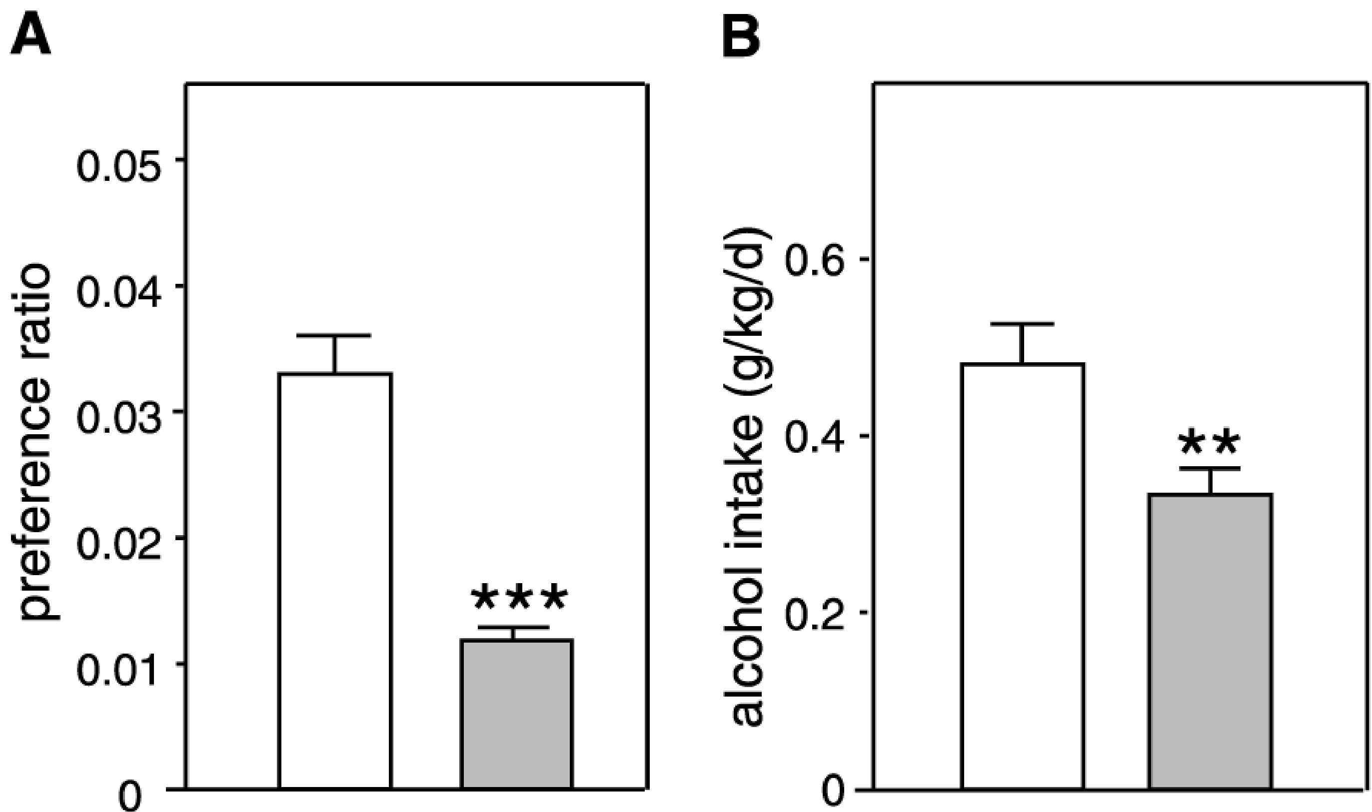

Figure 3. Alcohol consumption in TLM mice (filled bars) and their wild-type littermates (open bars) in a two-bottle choice paradigm. All values are means±SE. **A)** Mean alcohol preference ratio [proportion of the 10% (v/v) ethanol solution per total fluid consumption for 20 free choice 24-h drinking periods, ****P*<0.001]. **B)** Daily alcohol intake for 20 free choice 24-h drinking periods (***P*<0.01).

Fig. 4

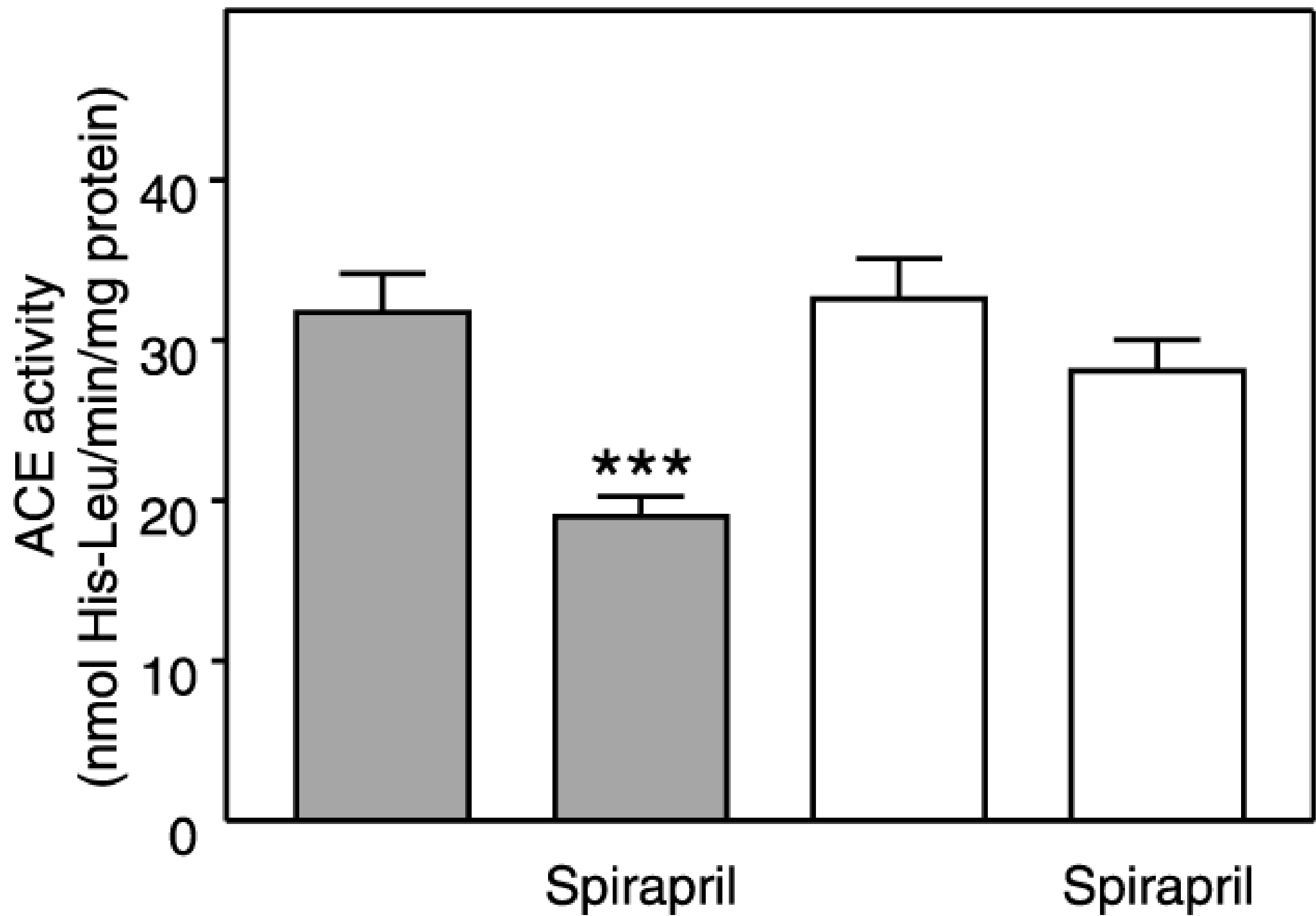

Figure 4. Specific ACE activity in brain membrane preparations. Homogenized mouse tissue (filled bars) and membrane preparations washed four times with buffer (open bars) before and after a daily spirapril treatment (10 mg/kg of body weight) for 10 days were assayed as described in the Materials and Methods section. ACE activity is given as the mean amount of the dipeptide histidyl-leucine generated per minute per milligram of total protein±SE (n = 24 for each column). Statistical comparison of the data for the unwashed samples revealed ***$P < 0.001$. The difference between the values of the buffer-washed samples was not significant.

Fig. 5

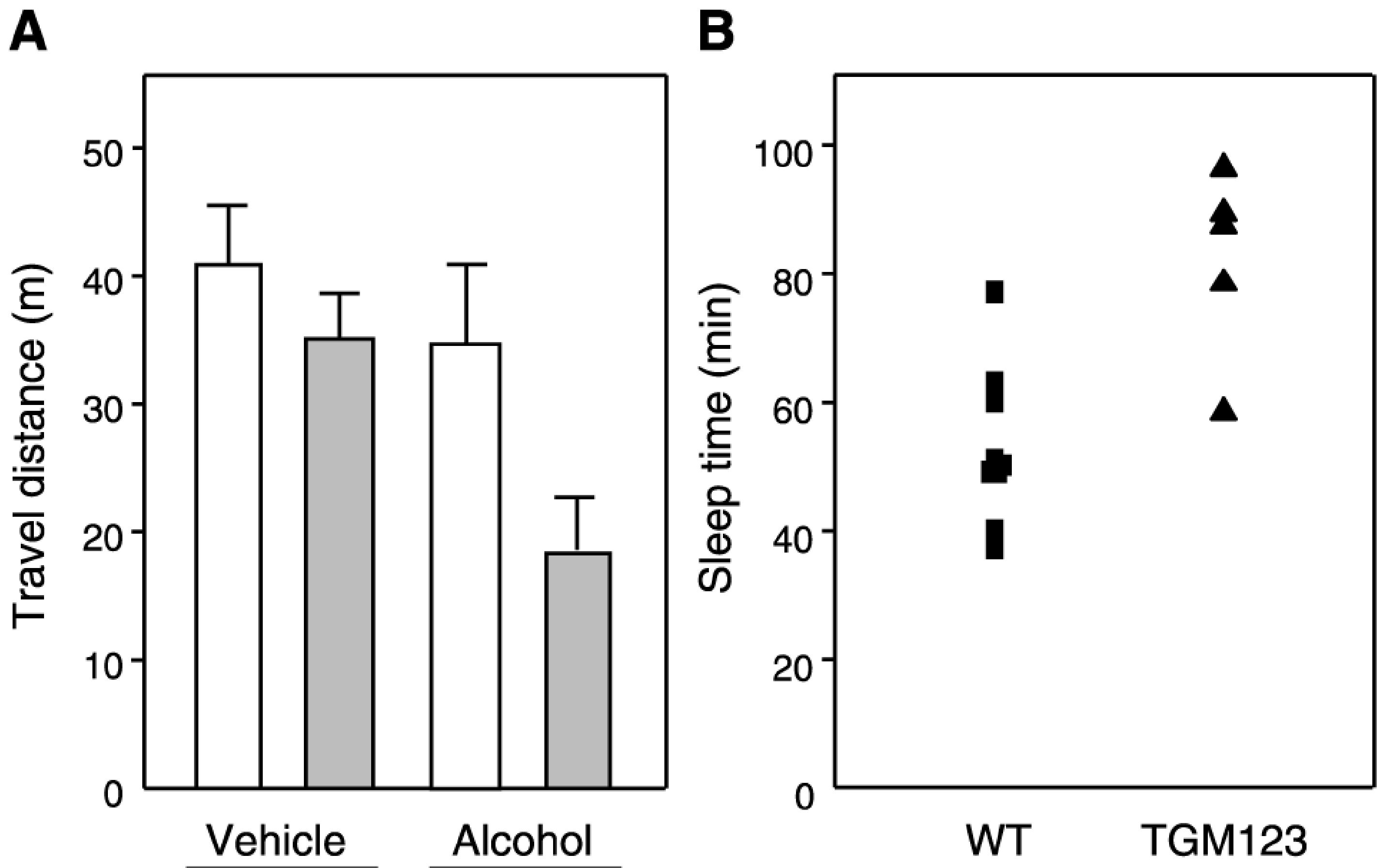

Figure 5. Central alcohol action in TGM123 mice. A) Horizontal activity of TGM123 mice (*n* = 8; filled bars) and controls (*n* = 11; open bars) in an activity meter. Mean travel distance in meters±SE after application of saline or ethanol at 2.5 g/kg of body weight during a 10-min test period. Interaction was found significant by a two-way ANOVA (treatment × genotype, $P<0.05$, $F = 4.644$). Post hoc analysis did not reveal a difference between the saline groups.
B) Sleep time after injection of ethanol at 4 g/kg of body weight in TGM123 mice (*n* = 6) and controls (*n* = 10). The time points mark the return of the righting reflex. Statistical comparison of the data resulted in $P < 0.01$ (Mann-Whitney U test = 3.0).

Fig. 6

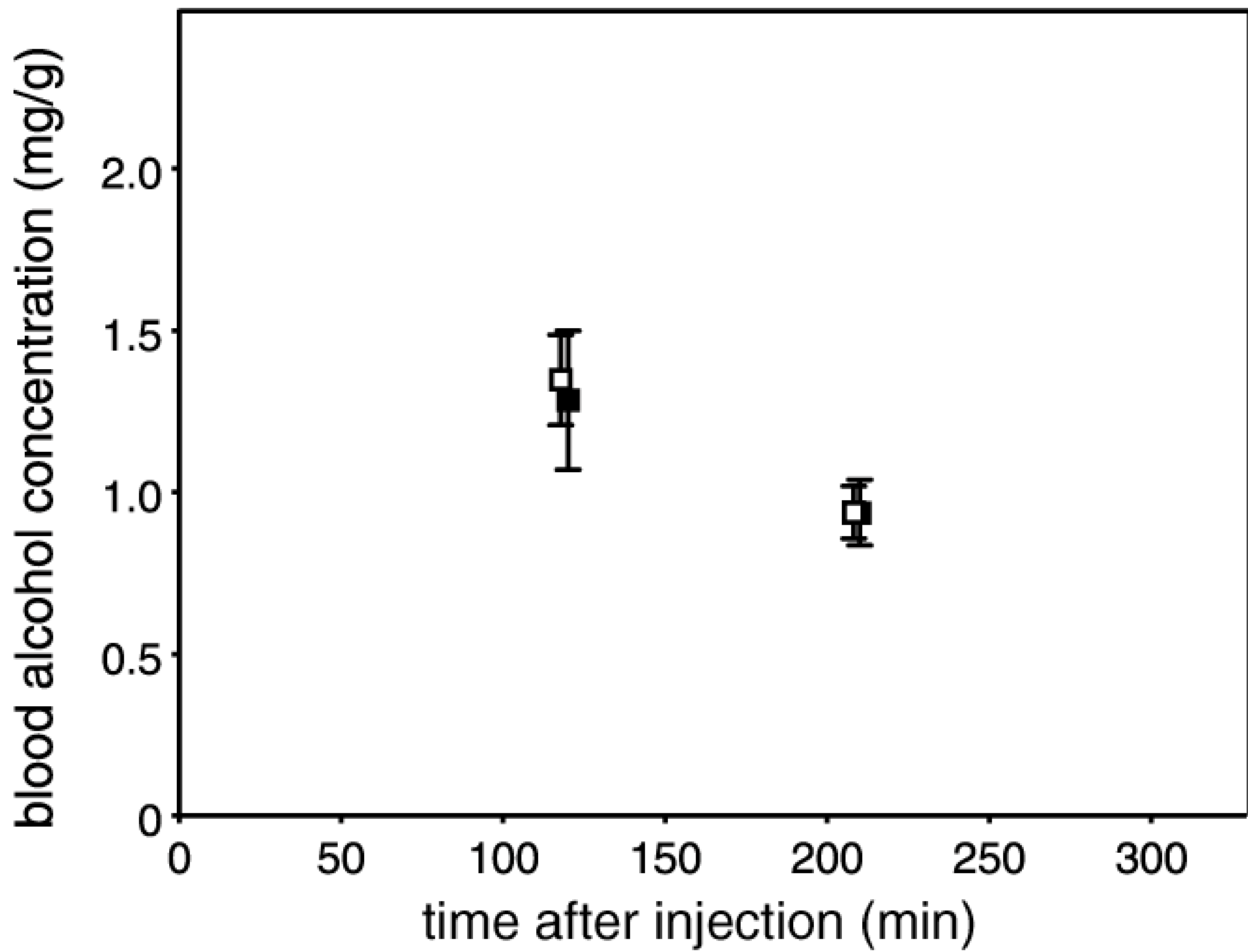

Figure 6. Alcohol degradation kinetics in TGM123 mice (filled boxes) and their wild-type littermates (open boxes) after i.p. alcohol injection (3.5 g/kg of body weight) at time t = 0.