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Neuronal Membrane Enzymes in Rat Lines Selected for Differential Motor Impairment by Ethanol

C. J. PETER ERIKSSON*[1] AND CONSUELO GUERRI†

Research Laboratories of the Finnish State Alcohol Company (Alko Ltd)
*Box 350, SF-00101 Helsinki 10, Finland**
and Instituto de Investigaciones Citologicas de la Caja de Ahorros de Valencia
Amadeo de Saboya, 4 Valencia-46010-Spain†

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ERIKSSON, C J. P AND C GUERRI. *Neuronal membrane enzymes in rat lines selected for differential motor impairment by ethanol* PHARMACOL BIOCHEM BEHAV 24(4) 1115–1121, 1986 —Neuronal membrane enzyme activities were determined in naive and ethanol-treated (30 min after 2 g/kg) male and female rats of lines developed for more (ANT) and less (AT) ethanol-induced motor impairment Ethanol did not affect acetylcholinesterase, (Na+K)-ATPase or 5'-nucleotidase activities, but adenylate cyclase activities were lowered in both cerebellum and cerebrum Cerebral acetylcholinesterase activities were higher in ANT than AT rats No consistent line difference was observed regarding (Na+K)-ATPase activities. Slightly higher cerebellar 5'-nucleotidase activities were found in the ANT line. Cerebellar adenylate cyclase levels were substantially higher in the AT line No line differences were displayed in the activation of adenylate cyclase activity by dopamine or norepinephrine It is concluded that ethanol *in vivo* may inhibit neuronal adenylate cyclase activity and that cerebellar phosphorylation may be a regulator of motor impairment. Cholinergic mechanisms may also be connected to the ethanol-induced motor impairment

Ethanol intoxication Motor impairment Genetically selected rat lines Acetylcholinesterase
5'-Nucleotidase (Na+K)-ATPase Adenylate cyclase

SELECTIVELY outbred animal lines provide an excellent basis for investigations of biological mechanisms which involve genetic variability In our laboratory, two rat lines have been developed for high (ANT=alcohol-nontolerant) and low (AT=alcohol-tolerant) degrees of ethanol-induced motor impairment as measured by the tilting-plane and rotarod tests [11,54]. In a previous study it was concluded that the genetically determined factors influencing motor impairment are for the most part dissociated from the factors determining the hypothermia and the narcotic effects of ethanol [9].

The aim of the present investigation was to approach the biochemical characterization of the mechanisms leading to differential ethanol-induced motor impairment in the ANT and AT rat lines. According to current belief, the neuronal membranes seem to include important sites for the production of ethanol intoxication. Thus, membrane enzymes should provide interesting targets for investigations of the genetically determined molecular mechanisms of ethanol-induced motor incoordination. For the first screening of membrane enzymes we chose brain adenylate cyclase, (Na+K)-ATPase, 5'-nucleotidase and acetylcholinesterase, which were examined in the ANT and AT lines with or without acute ethanol pretreatment.

METHOD

Naive, fed, male and female AT and ANT rats (2.0–2.5 months old) of generation F_{20}, and male rats (3.5–4.0 months old) of generation F_{21}, were used in the present investigation. Populations from both generations were divided into two subpopulations. One subpopulation, containing only males, was behaviorally tested. The other subpopulation, containing males and females, was used for biochemical determinations from brain samples. In the behavioral tests ethanol-induced motor impairment was measured by the tilting-plane, in which the difference in sliding angle before and 30 min after an ethanol injection of 2 g per kg of body weight was recorded [54]. Tail blood samples were taken immediately after the tilting-plane tests and ethanol concentrations were determined by headspace gas chromatography [10].

The animals from which brain samples were to be taken

[1]Requests for reprints should be addressed to C J Peter Eriksson, Ph D , Research Laboratories of the Finnish State Alcohol Company, Alko Ltd, POB 350, SF-00101 Helsinki, Finland

were divided into two groups, one of which received 2 g ethanol per kg of body weight (as 12%, w/v, in saline) The control rats received saline. Thirty min after ethanol administration the rats were sacrificed by decapitation, and brains were quickly removed. Brains taken from F_{20} animals were divided into the two lateral halves Both halves were subdivided into a cerebellar part (including lower brain stem) and a cerebral part (rest of the brain) Each cerebellar and cerebral part was homogenized in 10 parts (w/v) of cold 0 1 M glycylglycine, pH 7 5 (containing 1%, w/v, Lubrol PX and 3 mM DTT) with a Polytron homogenizer for 10 sec with cooling by immersion in an ice bath. These homogenates were used for the determination of adenylate cyclase by measuring the amount of cAMP formed from ATP by the cAMP binding assay [13] following earlier described procedures cAMP formation in incubation at 37°C, 5 min, containing 50 mM glycylglycine, pH 7.5, 5 mM ATP, 10 mM theophylline, 1 mM DTT, 0.1% BSA, 8 mM $MgCl_2$ and 0 01% tissue, blanks with ATP omitted were subtracted [27]

The other cerebellar and cerebral halves were homogenized with 10 parts of 0 25 M sucrose (containing 1 mM EDTA) as described above These homogenates were used for measuring acetylcholinesterase [7] and 5'-nucleotidase [39] One ml of these homogenates was treated with Na-deoxycholate (0.15%, w/v, final concentration) and was used to measure (Na+K)-ATPase incubation at 37°C, 10 min, containing 100 mM Tris-HCl, pH 7 4, 5 mM ATP, 5 mM $MgCl_2$, 100 mM NaCl, 15 mM KCl and 0 1% tissue, blanks, containing 10^{-3} M ouabain and Na^+ and K^+ omitted, were subtracted [17]. Inorganic phosphate formed in the 5'-nucleotidase and (Na+K)-ATPase incubations was measured colorimetrically [2]

Brains from F_{21} animals were quickly taken and cerebellum, cortex (dorsal and frontal), pons medulla, hypothalamus, striatum, and limbic forebrain were dissected out on dry ice and stored at −70°C After 4–5 months of storage the cerebral brain parts were thawed and homogenized as 2%, w/v, in ice cold 0 25 M sucrose (containing 1 mM EDTA) as described above These homogenates were used for acetylcholinesterase determinations. The cerebellums were frozen at −70°C until processed (two months of storage) Then, they were thawed and homogenized in 10 parts (w/v) of cold 0 1 M glycylglycine buffer, pH 7 5 (containing 3 mM DTT) using a Super Dispox Tissumizer at full speed for 50 sec with cooling by immersion in an ice bath This homogenate was used for determination of adenylate cyclase as described above Catecholamines (L-noradrenaline hydrochloride and dopamine hydrochloride) were dissolved in 40 mM Tris-HCl, pH 7 5 immediately before beginning the incubation assay

Proteins from all homogenates were determined by the Biuret method [51] Bovine serum albumin was used as a standard

RESULTS

The first part of the present investigation involved the F_{20} generation of the AT and ANT rats The following motor-impairment scores (difference in sliding angle before and after ethanol injection) from the tilting-plane test were obtained AT=5 9±4 9°, mean±SD (N=13) and ANT=17 8±7 0° (N=13) ($p<0$ 001, Student's t-test) No significant differences regarding body wt (AT=332±30, ANT=347±35 g) and blood ethanol concentrations (AT=47 2±4 9, ANT=45 5±6 5 mM) were observed. A line difference ($p<0.025$) was detected in the control tilting-plane test before ethanol administration, with the ANT animals showing slightly more coordination (AT=57 8±5 2, ANT=63 8±5 9, $p<0.025$) The sliding angles 30 min after ethanol injection decreased to 51 8±7 5° (−12%) in the AT rats and to 46 0±4 2° (−37%) in the ANT animals

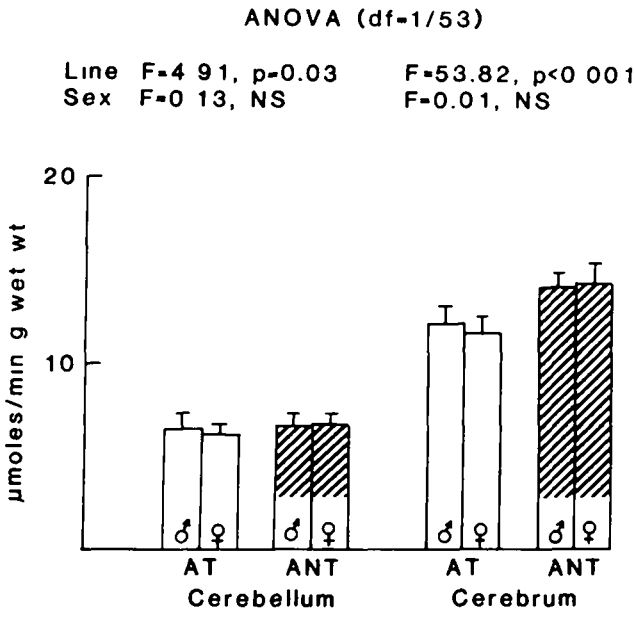

FIG 1 Cerebellar and cerebral acetylcholinesterase activity in F_{20} generation of the AT and ANT rat lines Bars express means±SD for control and ethanol groups combined (N=12–15)

The acetylcholinesterase results from the other F_{20} subpopulation are depicted in Fig 1 No effect of ethanol was observed and thus Fig 1 shows the control and ethanol data combined There was about twice as much activity in the cerebrum as in the cerebellum and a clear line difference (AT=14% (♂) and 17% (♀) <ANT, $p<0$ 001) appeared in the cerebral brain part Only a minor but nevertheless significant line difference occurred in cerebellum (AT=4% (♂)–10% (♀) <ANT, $p=0$ 03) No sex differences were observed Similar differences are obtained, if the results are expressed as specific activities

No line differences and no effects by ethanol on (Na+K)-ATPase activities were observed in either brain compartment. Combined control and ethanol means±SD (n=12–15) were for cerebellum 21 7±1 6 (AT ♀), 22 6±1 7 (ANT ♀), 22 8±1 7 (AT ♂), 20 9±1 8 μmoles/min g wet wt (ANT ♂), cerebrum 22 6±1 9 (AT ♀), 23 3±2 1 (ANT ♀), 21 9±2 1 (AT ♂) 19 8±1 4 μmoles g wet wt (ANT ♂) By using ANOVA, line/sex interactions were F=9 22, $p=0$ 004 (cerebellum) and F=7 35, $p=0$ 01 (cerebrum) If the sexes were dissociated, a line difference, with the activities of AT males being 9% (cerebellum) and 11% (cerebrum), ($p<0$ 01 and $p<0.005$, Student's t-test) higher than those of ANT males, was observed A sex difference (♂ =3% (AT) and 15% (ANT) <♀, $p<0$ 001) located in cerebrum was also noted As before, similar results were obtained also with the data expressed as specific activities

No ethanol effects were observed in the 5'-nucleotidase activities, combined means±SD (N=13–16) were for cerebellum 1 17±0.17 (AT ♀), 1.31±0 17 (ANT ♀), 1 14±0 12 (AT ♂), 1 19±0 17 μmoles/min g wet wt (ANT ♂), cere-

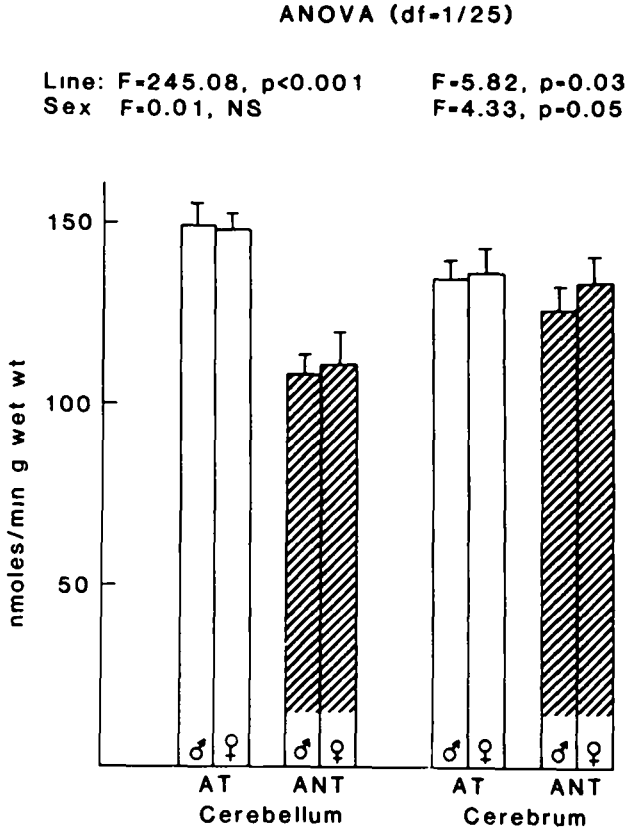

FIG 2 Adenylate cyclase Conditions are as for Fig 1 , except that only control data are expressed (N=6–8)

brum 0 86±0.09 (AT ♀), 0 91±0 11 (ANT ♀), 0.87±0.08 (AT ♂), 0.87±0.13 μmoles/min g wet wt. (ANT ♂). No line or sex differences were observed in cerebral material However, the cerebellar samples displayed small line (AT=4% (♂) and 10% (♀) <ANT, $p=0.02$) and sex (♂=3% (AT) and 9% (ANT) <♀, $p=0$ 05) differences. The cerebellar differences (AT=1–8% <ANT) were not statistically significant when calculated on the basis of specific units (per mg protein).

Figure 2 (control data) demonstrates the major adenylate cyclase differences in cerebellar samples (AT=33% (♀) and 38% (♂) >ANT, $p<0$ 001) and the minor differences in cerebral material (AT=5% (♀) and 14% (♂) >ANT, $p=0.03$) The corresponding results in the ethanol treated groups were: AT=45–47% >ANT, $p<0$ 001 (cerebellum) and no difference (cerebrum) The inhibitory effect of ethanol is also demonstrated in Fig. 3, which shows that the effect occurs both in cerebellum and cerebrum. Ethanol, however, appears to affect the cerebellum more in ANT rats (especially the females), but it affects the cerebrum more in the AT animals (Fig. 3). The cerebellar line difference was not significant (F=2 81) if absolute amounts of decrease were used for ANOVA. Expressing the results as specific activities had little effect on the adenylate cyclase differences

The second part of the present investigation involved the F_{21} generation It was given the same behavioral tests as the F_{20} generation, and the results (not shown) were essentially the same Only cerebral acetylcholinesterase and cerebellar adenylate cyclase, however, were chosen as biochemical pa-

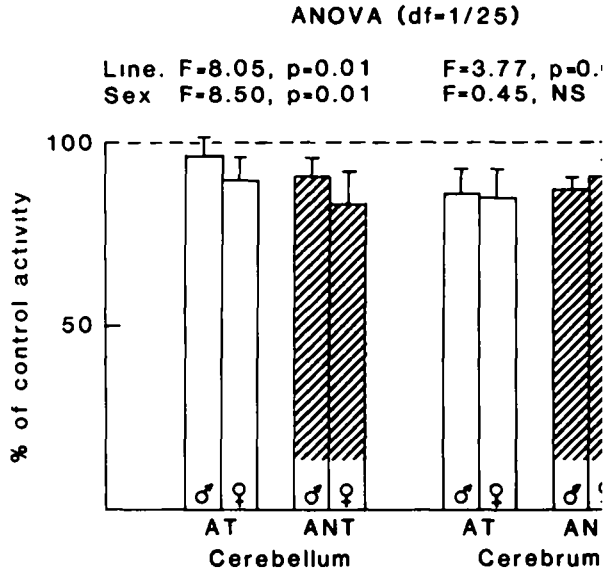

FIG 3 Effect of *in vivo* ethanol on adenylate cyclase activity C ditions are as in Fig 1, expect that ethanol and corresponding c trol values are separated (N=7–8)

rameters for further study. Figure 4 demonstrates the ce bral distribution of acetylcholinesterase most of the enzy was located in striatum and limbic forebrain, and the AT r displayed significantly less activity in these regions and cortical areas Ethanol treatment had no effect on the act ties

As observed with the F_{20} generation, higher cerebe adenylate cyclase activities were found in AT than in A rats (Fig. 5). An inhibitory effect of the *in vivo* ethanol tr ment was also observed again (AT=−8%, ANT=−16 The line difference was not significant ($t=1$ 98) if absol amounts of decrease were used for Student's t-test. Th were some procedural differences with the F_{20} and studies (Figs 2 and 5): the cerebellums of F_{21} rats were f of brain stem, they were frozen and stored, and their p duction of cAMP was measured in a detergent-free incu tion. The generally lower activities obtained with the generation may mostly be explained by the fact that de gents increase the production of cAMP [27,45].

In additional experiments, the stimulation of adeny cyclase by dopamine and norepinephrine was tested. No l differences were found (Fig. 6), nor were there any dif ences produced by ethanol treatment in the stimulated ce bellar preparations: if effects were calculated as percen control (nonstimulated) activities. If, however, absol amounts of activity changes were used for statistical cal lations, significant line differences ($F_{line}=12.57$, $p<0$ C occurred, with dopamine stimulating less the ANT adenyl cyclase activity.

DISCUSSION

Acetylcholinesterase

Acetylcholinesterase is responsible for the degradatio acetylcholine and is mainly concentrated on the postsyna membrane, where it terminates the action of acetylchol on the postsynaptic receptor [38]. Previous studies have

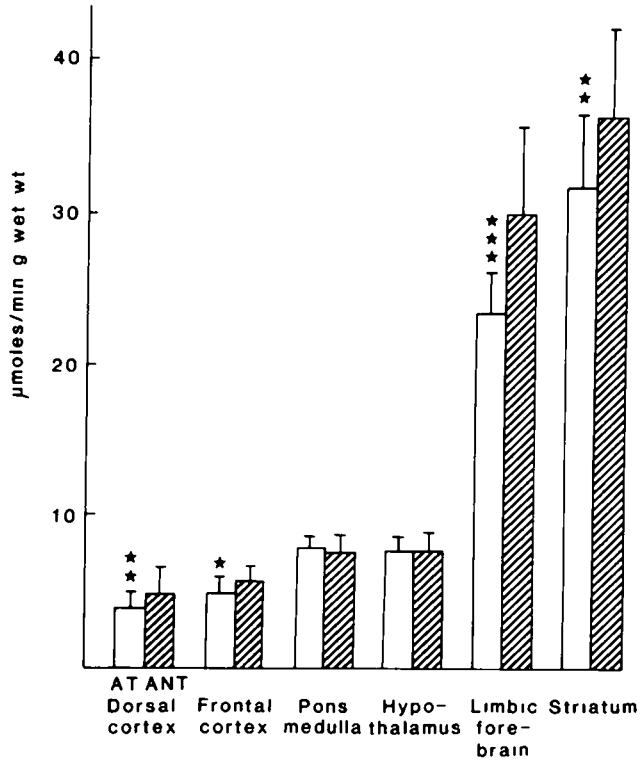

FIG 4 Cerebral distribution of acetylcholinesterase activity in generation F_{21} Bars express means±SD for control and ethanol groups combined (N=11–13) Significant line differences (Student's *t*-test) are denoted by ★$p<0$ 01, ★★$p<0$ 005, ★★★$p<0$ 001

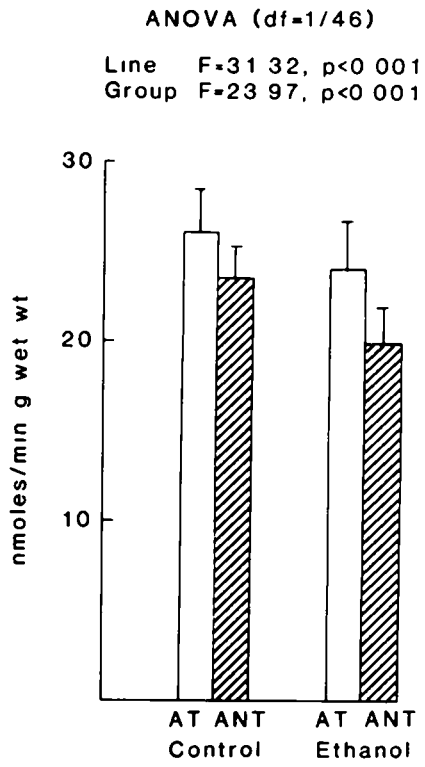

FIG 5 Effect of *in vivo* ethanol on cerebellar adenylate cyclase activity in generation F_{21} Bars express means±SD for control and ethanol-treated groups (N=12–13)

ported ethanol-induced inactivations [56], biphasic effects depending on ethanol concentration [59], or no effects [31], in different brain preparations *in vitro* In contrast to the earlier *in vivo* findings [32,44], our present findings, as well as some other recent mouse data [6,22], revealed no effects by acute ethanol on acetylcholinesterase activity This does not, of course, exclude an effect on the functional activity *in vivo*, which was no longer present during the *in vitro* condition when the *in vivo* ethanol concentration was diluted in the incubations.

The involvement of cholinergic systems in the mechanisms of motor-impairment by ethanol is, in fact, supported by the present line differences in acetylcholinesterase (Fig. 1 and 6). The data suggest that increased acetylcholinesterase activity, i.e , decreased levels of free acetylcholine and thus decreased cholinergic transmission, promotes the motor impairment This hypothesis is in line with previous general findings of ethanol blocking neuronal acetylcholine release [4, 8, 30, 31, 40, 46].

(Na+K)-ATPase

Na^+, K^+-dependent ATPase is associated with the plasma membrane and its main task is to participate in the energy-requiring translocation of sodium and potassium [28] That inhibition of this enzyme could be involved in the mechanism of ethanol intoxication is supported by several *in vitro* studies, in which ethanol, at least at sufficiently low potassium concentrations, inhibits neuronal enzyme activity [1, 12, 25, 29, 34, 36, 37, 52, 56, 58, 60]. Whether such an inhibition occurs *in vivo* is uncertain. Findings of both increased [24,26] and decreased [17] (Na+K)-ATPase activities by ethanol *in vivo* have been reported Our present finding, as well as other earlier data [19], revealed, however, no changes in activity in animals treated with acute ethanol compared with untreated controls. Again, as discussed above with the acetylcholinesterase, little can be said about possible functional activity changes *in vivo* based on *in vitro* incubations in which the ethanol concentration is minimal

The involvement of (Na+K)-ATPase in the mechanism of motor-impairment by ethanol, perhaps in a sex-linked manner, is suggested by the significant sex-line interactions observed in present study. The finding, at least in males, that higher basal ATPase activity may be associated with less sensitivity to ethanol-induced motor-impairment, is in line with the results of another earlier rat line comparison [41] No ATPase differences have been observed in mice lines selected for different durations of ethanol-induced loss of righting reflex [37].

It should be pointed out that in the present investigation only total (Na, K-dependent, Mg-activated) ATPase activities were measured. Future studies, measuring the (Na+K)-ATPase in different topographic areas of the brain, as well as in pure subcellular compartments, such as synaptic membranes, might be needed to characterize possible associations between impaired ATPase function and motor impairment

5'-Nucleotidase

5'-Nucleotidase catalyzes the production of adenosine from adenine nucleotides The activity of this enzyme has

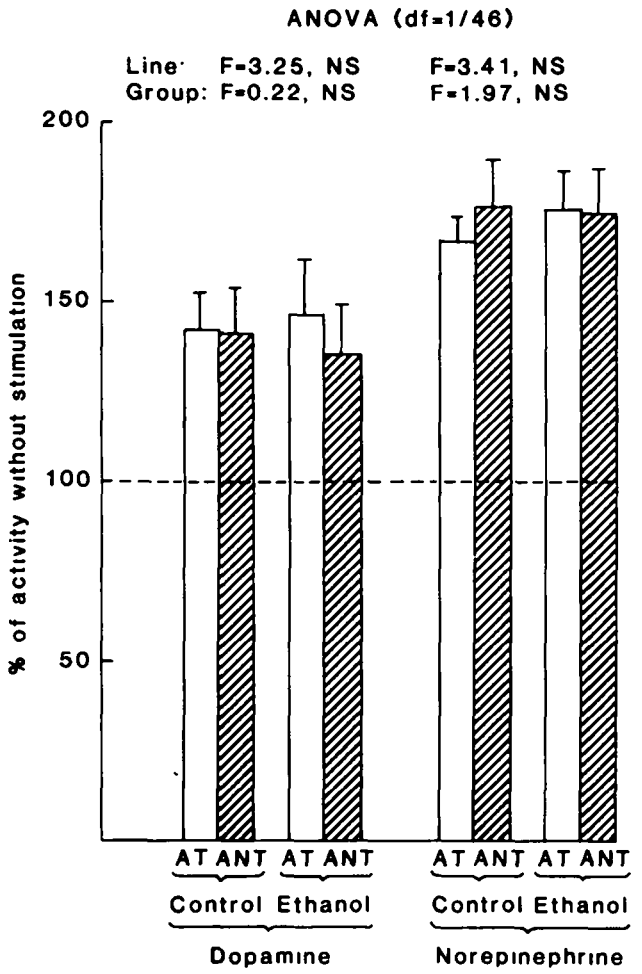

FIG 6 Effect of dopamine (50 μM) and norepinephrine (50 μM) on cerebellar adenylate cyclase activity *in vitro* in generation F_{21}. Bars express means±SD of the catecholamine values compared with corresponding values obtained without stimulation (N=12-13)

been suggested as a factor controlling free adenosine levels [55], and may thus affect adenosine-modulation of synaptic transmission. Little is known about possible ethanol-interactions with 5'-nucleotidase Chronic ethanol treatment has been observed to increase synaptosomal 5'-nucleotidase activities [16]. Acute, or *in vitro* effects, have not been reported. No effects by *in vivo* ethanol were found in the present study. However, the contribution of adenosine mechanisms in ethanol-induced motor impairment cannot be totally excluded, because of the tendency in the cerebellar material for higher basal 5'-nucleotidase activities in the ANT than in the AT rats.

Adenylate Cyclase

Adenylate cyclase is a membrane-bound enzyme, which catalyzes cAMP formation from ATP, the reaction which activates kinases and subsequent protein phosphorylation. Neuronal phosphorylation has, in turn, been suggested to be involved in certain synaptic transmissions [15]. In contrast to an early observation [33], several investigators have demonstrated that ethanol activates adenylate cyclase in different neuronal preparations *in vitro* [14, 20, 21, 35, 47, 48, 57, 62]. The mechanism of activation has been suggested to involve a physico-chemical interaction with membrane lipids in the microenvironment of adenylate cyclase.

No *in vivo* effect of ethanol has previously been reported The present results (Figs. 3 and 5), however, clearly demonstrate lower adenylate cyclase activity after an acute ethanol dose. These results, obtained in crude homogenates, are not in contradiction with earlier results of *in vitro* activation of adenylate cyclase in more purified systems. In fact, if ethanol was added *in vitro* to crude homogenates made without detergent (as for the present experiments with F_{21}) we also noted a dose dependent *in vitro* stimulation by ethanol (Eriksson, unpublished information). Thus, in spite of an activating effect *in vitro*, the net ethanol effect *in vivo* may be a reduction of cAMP formation and thus of phosphorylation. The mechanism of the *in vivo* inhibition is obviously not "washed away" during the preparation of the *in vitro* sample, and could perhaps involve the change in some of the modulators of adenylate cyclase activity. One important modulator *in vivo* is the calcium ion. The importance of this ion in the expression of the adenylate cyclase is well known, small concentrations are necessary for the basal activity, while large concentrations inhibit the activity [3]. Ethanol administration produces a depletion of the brain calcium [18,53]. Based only on the present *in vivo* inhibition, and on earlier results of *in vitro* activation, it is, of course, difficult to postulate the functional *in vivo* situation during ethanol intoxication. That inhibition is the net result *in vivo* is supported by studies showing lowered neuronal cAMP levels in animals under the influence of an acute dose of ethanol [42, 61, 62]. Other studies have, however, failed to observe significant *in vivo* cAMP changes [33, 49, 50], and the whole issue may have been hampered by methodological difficulties [23]. Biological variation may also explain the seemingly contradictory results, as perhaps demonstrated in a human study where ethanol in 10 subjects lowered, and in 1 subject increased, cerebrospinal cAMP [43]

The present line differences, with the more impaired ANT rats having lower cerebellar basal adenylate cyclase activities than the AT animals with low degree of intoxication, support the logic of the association between inhibited phosphorylation and intoxication. It should also be noted that the ethanol-induced *in vivo* inhibition of adenylate cyclase was higher (in percent) in the ANT compared with the AT animals and thus the ANT rats had much lower activities after ethanol than the AT animals did. Interestingly, this adenylate cyclase difference occurred only in cerebellum, thus suggesting that disturbed phosphorylation in this brain compartment may influence motor performance. In another recent study, no basal differences, but lower dopamine-stimulated cerebral adenylate cyclase activities were observed in mouse lines selected for shorter duration of loss of righting reflex compared with the more sensitive line [5]. Thus, until further studies have been made, it may be premature to assume in general that all components of ethanol intoxication involve inhibited phosphorylation

In order to get some indication as to which adenylate cyclases and which modulators were important in producing the present line differences regarding motor impairment, dopamine and norepinephrine stimulation experiments were performed *in vitro* The results of these experiments (Fig. 6) seem to suggest that the adenylate cyclase line differences were not related to the dopamine or norepinephrine receptor-coupled adenylate cyclases. On the other hand ANT adenylate cyclase was less stimulated if dopamine

stimulation was calculated as absolute amount of activity, which demonstrates how difficult it is to interpret results based on stimulation of already differing basal activities

Thus, these transmitter-coupled adenylate cyclases, as well as other transmitter and modulator mechanisms, should be examined more closely in future studies

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